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IN VITRO STUDIES OF NEUROTOXIC SUBSTANCES

DEPARTMENT OF BIOLOGY
CATHOLIC UNIVERSITY OF AMERICA
WASHINGTON, D.C. 20064

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An in vitro strategy for the evaluation of the neurotoxic potential of chemicals was developed. A number of investigations were undertaken to validate portions of the proposed test battery. The components of the test battery which were used are as follows: neuroblastoma cell lines and chick brain cell and organ culture tester cells; acetylcholinesterase, neuron-specific enolase, and neurotoxic esterase activity, and acetylcholine receptor number as

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neuronal end-points; acrylamides and organophosphates as model chemicals. Using these in vitro systems target specific toxicity of acrylamides for neuro-specific enolase and organophosphates for neurotoxic esterase and acetylcholinesterase were demonstrated. Furthermore, the toxicity ranking in vitro closely parallels the toxicity ranking of these chemicals in animals and humans. The research also led to the development of an in vitro alternative for the hen brain assay for neurotoxic esterase (which is predictive of delayed neuropathy), the validation of the use of phenyl 4-butyrate (which is commercially available) as a substitute substrate for phenyl valerate (which must be custom synthesized) in the neurotoxic esterase assay, and the development of an in vitro method for the evaluation of the efficacy of anti-organophosphate chemical defense agents, such as an anti-paraoxon monoclonal antibodies. Studies on acetylcholine receptors indicate that the receptor number in chick brain cultures increases with time in culture as well as with exposure to organophosphates.

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INTRODUCTION

The expanding use of *in vitro* systems for carcinogenesis, mutagenesis, and general toxicity studies attests to their potential and real value. Their use in screening, mechanistic studies, and personnel monitoring have provided benefits in cost, time, and control which often outweigh the limitations of the systems, provided they are used judiciously (Nardone, 1980).

The strong focus on in vitro genetic toxicology and carcinogenesis during the past decade has resulted in a clear understanding of the attributes and limitations of in vitro tests. Thus, regulatory agencies and industry now rely on batteries of such tests for the preliminary evaluation of the mutagenic and carcinogenic risk to be associated with chemicals.

An analysis of this developmental period provides us with insights which could be applied to the development of in vitro tests and to mechanistic studies related to other forms of toxicity, such as neurotoxicity. Table 1 summarizes the three stages in development of in vitro tests: the basic research period, the test validation period, and the period of secondary evolution (Nardone, 1981)

Recent advances in cell and tissue culture have resulted in the availability of an expanded repertoire of cell types suitable for toxicity testing in general and for the testing of target specific substances, such as neurotoxic agents, as well for mechanistic studies (Fedoroff and Hertz,1977; Trapp and Richelson, 1980). Among the significant advances in neuronal cell culture have been those which have involved the culture of continuously proliferating neuroblastoma and glioma cells which, in vitro, continue to manifest their differentiated properties. These include for neuroblastoma cells neurite formation, action potential generation, synapse formation, and specialized neurotransmission-related biochemical properties. Glioma cells in culture also show differentiated properties including the production of nervous system specific proteins and inducibility by catecholamine and cortisol.

Tissue and organ culture reaggregation studies with tissues such as brain have proved to be especially useful in the study of development, cell recognition, sorting and interconnection (Seeds, 1973). Organ cultures of spinal cord, dorsal ganglia, cerebrum, hippocampus, hypothalamus, and cerebellum have been successfully maintained in vitro. The success is illustrated by the work of Aparicio et al (1976), who observed that within 14 days after explantation, pieces of cerebellum showed "the basic organ features -- cortical

layering, synapse formation, and myelination of axons." Table 2. lists other examples.

In respect to neurotoxicology, the current state of the field is "early basic research period." While the growth and other culture requirements of primary cells, established cell lines, organotypic cultures, and three-dimensional aggregates are known (IA of Table 1) very few toxicology investigations have exploited these. However, a few reports indicate that the potential for screening and mechanistic studies is very promising (see Spencer and Schaumberg (1980) for representative examples).

Among the neurotoxic agents are chemicals used in pest control and manufacturing processes, such as the organophosphates and carbamates (Davis and Richardson, 1980). In view of their economic importance and the serious health implications stemming from exposure to these chemicals, they warrant careful monitering and understanding regarding their mode of action. Only in that way can the public be protected and can we gain those insights which are required for the development of preventative and/or therapeutic approaches related to anticipated or actual exposure. It was with this in mind that this research was proposed.

Initially, the objective of this proposed research was to develop and validate a battery of in vitro tests for the evaluation of diverse neurotoxic substances, including organophosphates. Later, it occurred to us that the same in vito tests could be used to evaluate the efficacy of protective agents and putative therapeutic approaches.

TASKS AND UNDERLYING RATIONALE

The overall assessment approach for validation of the in vitro model is designed to enable one to distinguish between general

(non-specialized) toxicity and neurotoxicity. Hence, comparisons were to be made between cells of non-neuronal origin and those of neuronal origin and between inhibition of non-neuronal end-points (e.g. growth) and neuronal end-points (e.g. acetylcholine receptor synthesis). Second, the gene-tox studies have demonstrated that no one cell or test will satisfy all needs. Hence, it was expected that studies were to be undertaken to identify the best cells and tests for specific classes of chemicals, thus leading to the constuction of efficient test batteries. Furthermore, the validity of any emergent battery of tests as predictors of the risk which may be encountered by exposure of human and animal populations must show good in vitro and in vivo correlations. Table 3 summarizes the assessment approach which guided this project.

1. Because many organophosphates produce toxic intermediaries when they are metabolized by mixed function oxidases (Davis and Richardson, 1980) and because of their lipophilic nature, toxicodynamic studies must be undertaken at the onset. These studies were to involve the use of S-9 or microsome preparations from the livers of Aroclor-induced rats (Kouri and Schectman, 1976) and rat hepatocytes.

2. Two non-neuronal cell lines (L and IMR-90) and two neuronal cell lines (N1E-115 and a neuroblastoma x glioma hybrid cell line NG-108-15 were selected for use in the initial toxicity experiments. Strain L is an extensively studies established fibroblast cell line from mouse. IMR-90 is a human diploid cell line which is being exploited as a replacement for WI 38, the supply of which is becoming limited. Both cell lines show contact inhibition and inhibition by serum deprivation, thereby permitting studies on

proliferating and non-proliferating populations. NIE-115 is a clone of the C1300 mouse neuroblastoma cell line. It shows many neuronal properties including transmission enzyme synthesis, neurite formation, acetylcholinesterase production, and acetylcholine receptors (Nelson, 1977). Furthermore, its phenotypic expression can be modulated with cAMP, glial factor, nerve growth factor, or serum deprivation resulting in the favored expression of neuronal properties such as increase levels of tyrosine hydroxylase, and acetylcholinesterase, and synapse formation with co-cultivated myoblasts. Thus, comparisons could be made between non-neuronal and neuronal states using the same cell line and serum deprivation could be a common element when non-proliferating neuronal and non-neuronal cells are compared.

3. The assessment approach outlined in Table 3 was developed. The neurotoxic chemicals selected for study are varied in their neurotoxicity and have known mechanisms of action.

Non-neuronal end-points to be studied were cell viability (by trypan blue exclusion), cell proliferation by growth determinations and/or plating efficiency determinations, and protein synthesis.

Neuronal end-points to be assayed originally included tyrosine hydroxylase, acetylcholine receptor assays by α-bungarotoxin binding (Vogel and Nirenberg, 1976), and acetylcholinesterase activity levels. Tyrosine hydroxylase was dropped and neurotoxic esterase, total esterase, and neuron specific enolase activities were included.

4. Cytopathology caused by the organophosphates was assessed by light and transmission electron microscopy in selected instances.

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Table 1.

STAGES IN THE DEVELOPMENT OF *IN VITRO* TESTS FOR TOXICOLOGICAL EVALUATION

I. BASIC RESEARCH PERIOD

- A. NERVE CELL, TISSUE AND ORGAN CULTURE
  - 1. NUTRITIONAL AND MAINTENANCE REQUIREMENTS
  - 2. CHARACTERIZATION OF BIOLOGICAL MATERIAL IN RESPECT TO DIFFERENTIATED FUNCTIONS
- B. TOXICOKINETIC AND TOXICODYNAMIC STUDIES
  - 1. TRANSPORT AND EXPOSURE RESTRICTIONS
  - 2. BIOTRANSFORMATION COMPETENCE
- C. IDENTIFICATION AND PRELIMINARY PROBES OF SELECT END-POINTS
  - 1. *IN VITRO* - *IN VIVO* CORRELATES
  - 2. NEURONAL - NON NEURONAL CORRELATES

II. TEST VALIDATION PERIOD

- A. STANDARDIZATION OF TESTING PROTOCOLS
- B. TESTING OF SYSTEM WITH KNOWN POSITIVE AND NEGATIVE CONTROLS
- C. TESTING OF DIFFERENT CLASSES OF CHEMICALS
- D. FALSE POSITIVES, FALSE NEGATIVES AND CERTITUDE
- E. DESIGN AND VALIDATION OF TEST BATTERIES

III. PERIOD OF SECONDARY EVOLUTION

- A. INTER - AND INTRA - LABORATORY CONCORDANCE AND DISCORDANCE
- B. RESTANDARDIZATION OF TESTING PROTOCOLS
- C. ADOPTION BY INDUSTRY AND/OR FEDERAL AGENCIES

Table 2. The Components of the Test Battery

1. Mouse neuroblastoma NIE-115
2. Neuroblastoma x glioma hybrid cell line NG108-15
3. Chick brain cortex organ culture
4. Chick brain cell aggregate
5. Mouse primary spinal cord culture

Table 3. Neuronal and Nonneuronal End Points Used in the Test Battery

Neuronal End Points

1. Neuron-specific enolase activity
2. Neurotoxic esterase activity
3. Acetylcholine esterase activity and localization
4. Acetylcholine receptor number
5. Microtubular organization in neurites
6. Brain aggregate topography and neuronal and glial cell distribution

Nonneuronal End Points

1. Cell viability
2. Plating efficiency
3. Cell proliferation
4. Macromolecular content and synthesis rates (DNA, RNA, and protein)
5. Total enolase activity
6. Total esterase activity

2. In Vitro Effects of Acrylamides on Cellular Toxicity and Neuron-Specific Enolase Activity

2.1. INTRODUCTION

The expanding use of *in vitro* systems for carcinogenesis, mutagenesis, and general toxicity testing attests to their potential and real value and ever-increasing role in monitoring the quality of our environment. Expectations associated with the use of *in vitro* systems include benefits in cost, time, and understanding (Nardone, 1980).

Despite the fact that mammalian cell cultures have been used for toxicity studies for about two decades, the field is still plagued by a number of inadequacies. These include, until 1975, a lack of any systematic studies aimed at the standardization and validation of *in vitro* test systems, and a failure to exploit the opportunity presented by the dramatic advances in tissue culture techniques and somatic cell genetics made during the past decade. These advances include some which offer great promise for the attainment of an expanded repertoire of cell types suitable for toxicity testing in general and for the testing of target specific substances, such as neurotoxic agents which may be of special interest to those responsible for monitoring the environment.

Among the neurotoxic agents are chemicals which of military interest or are used in pest control, such as organophosphates and carbomates, as well as substances related to the manufacture of plastics and other materials. Casida *et al* (1976) have reported that over 200 million pounds of organophosphorus compounds are produced world-wide each year for biocidal purposes.

In view of the economic importance of these chemicals, continued attempts are being made to develop analogs which are less neurotoxic to mammals and, in the case of pesticides, more toxic to insects.

Validated *in vitro* test systems developed specifically for the assessment of neurotoxicity in its many forms could be useful aids in the evaluation of such substances.

Cell, tissue, and organ culture studies involving materials of neuronal origin have been employed since Harrison made his classical observations on nerve cells in tissue culture in 1907. Among the significant advances in neuronal cell cultures have been those which have involved the culture of continuously proliferating neuroblastoma and glioma cells which, *in vitro*, continue to manifest their differentiated properties. These include for neuroblastoma cell neurite formation, action potential generation, synapse formation, and specialized neurotransmission-related biochemical properties. Glioma cell in culture also show differentiated properties including the production of nervous system specific proteins and inducibility to catecholamine and cortisol.

Tissue and organ culture reaggregation studies with tissues such as brain have proved to be especially useful in the study of development, cell recognition, sorting and interconnection (Seeds, 1973). Organ cultures of spinal cord, dorsal ganglia, cerebrum, hippocampus, hypothalamus, and cerebellum have been successfully maintained *in vitro*. The success is illustrated by the work of Aparicio *et al* (1976), who observed that within 14 days after explantation, pieces of cerebellum showed "the basic organ features -- cortical layering, synapse formation, and myelination of axons".

The facility with which replicate cultures of genetically homogeneous cells can be maintained as monolayers, and the availability of clearly defined nervous system end-points in such cultures, suggest strongly that the development of model systems for the testing of neurotoxic substances should revolve strongly around monolayer

cultures of replicating (transformed) cells. These should be complemented by the use of primary cell cultures and brain aggregate and organ culture systems for those studies which may require endpoints not manifested by monolayer cultures.

The development of a model system for testing xenobiotics requires extensive validation. For such purposes related and unrelated chemicals whose in vivo toxicity ranking is known, are employed. Ideally, the specific mode of action of the test chemicals should also be known. Acrylamides and organophosphates satisfy these criteria (at least in part) and have been selected as the test chemicals used in the initial validation. Because of the greater economic importance of the organophosphates and the larger data base regarding their diverse biological effects, they constitute the chemicals of choice for most of the research which was undertaken for this project.

The organophosphate (OP) compounds represent a diverse group of chemicals widely used as pesticides, lubricants, hydraulic fluids, plasticisers, and flame retardants (Johnson, 1980). Acrylamides represent another class of neurotoxic chemicals which are widely used and diverse in their application. Since the early 1950's acrylamide has been used as a flocculator to strengthen paper and carboard, as a grouting agent, as a separating menstrum, and for many other purposes (McCollister et al, 1964). In 1972, 35 million pounds of the polymer and 50 million pounds of the monomer were produced in the United States (Spencer and Schaumberg, 1974). Soon after its introduction, the neurotoxicity of the monomer became recognized among industrial workers (Fujita et al, 1960). Low exposure led to drowsiness and a lack of concentration while higher exposure was accompanied by distal numbness and weakness. In animals, acrylamide caused ataxia and seizures.

Fullerton and Barnes (1966) reported that acrylamide causes peripheral nerve distal neuropathy. Such a dying-back pattern of damage is similar to that associated with organophosphorus-type degeneration.

The toxicity of acrylamide can be reduced by molecular changes. Hence, while the LD_{50} for acrylamide (AA) dosed rats is 1.5 mmol/kg, the LD_{50} for N-methylacrylamide (NMA) and crotonamide (CA) are 5.6 and 32 mmol/kg, respectively (Hashimoto et al, 1981). The toxicity may stem from several factors, most notably the inhibition of neuron specific enolase, a nerve-specific form of enolase, and other glycolytic enzymes, such as glyceraldehyde-3-phosphate dehydrogenase and phosphofructokinase (Howland et al, 1980a,b).

Hence, the acrylamides and the organophosphates represent good candidates to serve as model compounds for the development and validation of an in vitro model for neurotoxicity. Both are neurotoxic. The classes include analogs whose in vivo neurotoxicity ranking is known and differs from compound to compound. The mechanisms of toxicity is understood, at least in part. Thus, molecular targets have been identified.

2.2 OBJECTIVES:

The objectives for the first part of this research program were as follows:

1. To establish the cell and organ culture systems to be used for testing. For the first year, these include a chick brain cortex organ culture, an established cell line with neuronal properties (NIE-115) and a non-neuronal cell line (Strain L).

2. To establish the assays for monitoring neuronal and non-neuronal end-points which will yield information regarding neuronal and general cytotoxicity, respectively.

The neuronal end-points which have been selected include neurotoxic esterase, neuron-specific enolase, and acetylcholinesterase, and microtubule formation in neurites. The non-neuronal end-points include macromolecular synthesis, cell viability, plating efficiency, enolase activity, and esterase activity.

3. To determine the relative toxicity of acrylamides and several organophosphates in these systems.

2.3 METHODS

Cell Aggregation and Cortex Organ Culture

Cerebral cortices were aseptically dissected from the corpus striatum of 10-day white leghorn chick embryos; staged according to Hamburger & Hamilton (1951). The cortices were freed from their meninges, and washed in sterile Tyrode's solution. For aggregation studies, the cortices were washed three times in Calcium and Magnesium-free Tyrode's solution, (CMF), and prepared for dissociation according to the procedure of Moscona (1961). Briefly, this involves incubation in 0.67% trypsin (Grand Island) solution at 37°C for 20 minutes in a 5% CO_2-95% air atmosphere. After 3 washes with CMF, control culture medium (Medium 199, supplemented with 1% L-glutamine, 50 units/ml each of penicillan and streptomycin; and 25 ug/ml DNAse I)were added, and the tissue was mechanically dissociated into single viable cells by repeated flushing through a fine-bore pasteur pipette. Approximately $1x10^7$ cells (cells from three cortices) were dispensed into 3ml of the control or test medium contained within a 25ml erlenmeyer flask. The flasks were sealed in a 5% CO_2 -95% air environment, and incubated at 37.5°C in a rotary shaker ($\frac{3}{4}$ inch orbit of rotation) at 70 RPM.f Organ culture of 10-day embryonic chick cortex were similarly incubated on the rotary shaker in 3ml of medium minus the DNAse.

Acetylcholinesterase Assay

Cortex organ cultures or aggregates (pelleted) were homogenized in 0.1M Phosphate buffer (pH 8.0), and assayed for acetylcholinesterase activity using the colorimetric method of Ellman et al (1961). Using acetylthiocholine as the substrate, enzymatic hydrolysis yields thiocholine, which forms a color complex with 5,5[1] Dithiobis-2-nitrobenzoic acid. Specific activity of acetylcholinesterase is expressed as nanomoles of acetylthiocholine hydrolyzed per

minute per mg protein or as nonomoles of acetylthiocholine hydrolyzed per minute per cortex (for organ culture) or per cortex equivalent (for aggregate cell culture). True acetylcholeresterase activity is distinguished from total cholinesterase activity by the addition of 80 μM tetraisopropylpyrophosphoramide, a selective irreversible inhibitor of non-specific cholinesterase. Protein determinations were made by the method of Lowry et al (1951).

Morphological Appraisals of Cortices:

Cortices were fixed using 3% paraformaldehyde -- 2.8% glutaraldehyde in 0.05 M phosphate buffer pH 7.5, post-fixed for 3 hours in 1% osmium, dehydrated through alcohol and acetone, and embedded in Spurr. One micron sections were stained with azure-methylene blue. Thin sections were prepared as follows: hardened blocks were trimmed and sectioned using a diamond knife to obtain thin sections. Thin sections were stained with both lead and uranium, and examined at 60 kV in a Zeiss Electron Microscope. For light micoscopy, 1 μm sections were cut using glass knives and stained with toluidine blue.

Electron Microscopy of Neuroblastoma Cells:

In order to preserve the characteristic morphology of the neuroblastoma displayed in monolayer, cultures were fixed initially *in situ* (Goldman, 1972). Initial fixation was in 2% glutaraldehyde, 0.1 m cacodylate buffer, pH 7.2. After approximately 20 minutes in this fixative the cells were gently scraped from the growth surface using a rubber policeman. Fixation was allowed to continue in glutaraldehyde for a total of 45 minutes. At this point cells were pelleted and processed as a pellet through subsequent procedures. Post-fixation was in 1% OsO_4 in 0.1 M cacodylate buffer, pH 7.2 for 30 minutes. Cells were stained *en bloc* with 0.5% aqueous uranyl acetate, dehydrated through an acetone series and embedded in Spurr's epoxy resin mixture (Spurr, 1969). Polymerized blocks were sectioned using a DuPont diamond knife and a Reichart

ultramicrotome. Sections were stained with lead citrate (Reynolds, 1963) and examined at 60 KV using a Zeiss Electron Microscope.

Cell Culture Methods, Cytodifferentiation and Neurotoxic Esterase (NTE) Assays:

Cell culture methods described below for NTE studies were also employed for studies on acetylcholinesterase and neuron-specific enolase inhibition by acrylamides.

Intact cultures of differentiated neuroblastoma C-1300 clone NIE-115 (Amano et al, 1972) are used in place of whole animals. Cell culture homogenates are used in place of hen brain homogenates. Cell line NIE-115 was kindly provided by M. Nirenberg of the NIH. Cell cultures were grown as monolayers in Corning 75 cm^2 polystyrene flasks (Corning Glass Works, Corning, N.Y.), with Dulbecco's modification of Eagle's minimum essential medium (DMEM), obtained from Flow Laboratories (McLean, Va.). Media was supplemented to a 10% concentration with newborn bovine serum (Flow) and buffered with 3700 mg/L of sodium bicarbonate. Cultures were maintained in an environment of 10% CO_2 - 90% air at 37°C (Air Products, Hyattsville, Md.). These cells possess the capability to differentiate morphologically, biochemically, and electrophysiologically in the presence of cAMP. Cell cultures were differentiated by withdrawal of serum and addition of 0.5 mM dibutyryl adenosine 3', 5'-cyclic monophosphate (cAMP) from Sigma when cells had reached stationary phase. All OP stocks were prepared at a 20 mM concentration in DMSO (Fisher Scientific Company) and added to whole cultures or homogenates as indicated.

The following organophosphate compounds were obtained from the Environmental Protection Agency Analytical Reference Standards Repository, Raleigh, N.C.: O-methyl O-4 bromo-2, 5-dichlorophenyl phenylphosphorothioate (leptophos), O-ethyl O-4-nitrophenyl phenylphosphorothiate (EPN), actomethyl pyrophosphoramide (OMPA), O, O-diethyl O-P-nitrophenyl phosphate (paraoxon). All reference standards were greater than 98% pure. N, N diisopropylphosphorodiamidic

flouride (mipafox) and phenyl valerate were kindly provided by M.B. Abou-Donia, Duke University. DFP was obtained from Sigma. Leptophos-oxon was also obtained from the EPA. After exposure of cultures to OP's in situ the cells were rinsed in saline, scraped, homogenized in a 7 ml Wheaton Dounce for 20 strokes, and assayed according to the method of Johnson (1977) for NTE with necessary modifications. This method is a differential assay requiring the two OP's paraoxon and mipafox to define the amount of NTE present. Paraoxon (50 μM) is added to all test samples except for a control to inactivate irrelevant esterase activity. Mipafox (200 μM) is then added to a sample. Inhibition of esterase activity beyond that inhibited by the paraoxon is NTE. Any inhibition of a test OP beyond that due to the paraoxon is defined as inhibition of NTE. Johnson (1975) found phenyl valerate to be a more specific and sensitive substrate than phenyl phenylacetate for measuring NTE so this is now the substrate of choice. Esterase activity is measured spectophtometrically at a wavelength of 510 nm. Phenol red is found by the hydrolysis of the substrate, hence the more activity the higher the O.D. 510.

2.4 Experimental Results:

Dose Response to Acrylamide: Chick Brain:

Dissociated 10-day embryonic chick cerebral cortex (C x C^{10}) cells were aggregated for 24 hours in serum-free control medium and compared to C x C^{10} cells aggregated in 1, 3, 5, 10 and 15 mM acrylamide. One mM acrylamide had no effect on the size or morphology of 24 hour aggregates. Three and five mM acrylamide affected a slight reduction in aggregate size, but the aggregate morphology was still compact and similar to the control aggregate. Eight mM acrylamide elicited a definite effect upon aggregate morphology. The aggregates were smaller, less compact, more flattened, and much more irregular in shape. This partial inhibition of aggregate formation was enhanced with 10 mM acrylamide exposure. Cultures aggregated in the presence of 10 mM acrylamide were markedly smaller; with irregular aggregate contours or shapes. Fifteen mM acryalmide caused a complete inhibition of cellular aggregation.

Kinetics of Aggregation Inhibition by 10 mM Acrylamide:

The initial 2 hours of aggregation were similar to control cultures. The first indication of an effect of 10 mM acrylamide on C x C^{10} cell aggregation was at 3-4 hours; taking the form of aggregate clusters with slightly less regular contours. The toxic response of 10 mM acrylamide was markedly evident by approximately 6 hours of aggregation. By this time, control aggregates were initiating the process of compaction into definitive spheroid shapes with regular contours. By contrast, aggregate clusters in the presence of 10 mM acrylamide remained smaller and did not become compact.

In control cultures, the time period from 6 - 10 hours of aggregation involved a period of aggregate growth (primarily via secondary fusions of primary aggregates followed by compaction). Aggregates in the presence of 10

mM acrylamide also enlarged during this time, but the aggregate fusions were more "agglutination-like"and were not followed by condensation into compact aggregate forms.

During the time period from 10 - 24 hours of aggregation, control aggregates continued to secondarily fuse and compact into their definitve spheroid aggregate shape. Aggregates cultured in the presence of 10 mM acrylamide deteriorated from their condition at 10 hours of culture, becoming the characteristic small, loose, flattened, irregular shaped cluster aggregates by 24 hours.

To test if there was a particular time during early aggregation which was critically sensitive to the toxic effects of acrylamide, I administered acrylamide (to a final concentration of 10 mM) to cultures at various time points (2, 3, 4, 7, 8 1/2, and 10 hours) after the onset of aggregation in control medium. Regardless the time of acrylamide administration, the aggregates at 24 hours of culture were grossly affected in both size and appearance.

Aggregation of C x C^{10} Cells Pre-exposed to Acrylamide as Organ Cultures:

Taking advantage of the thin sheetlike nature of the embryonic chick cortex, I exposed the intact cortices to 10 mM acrylamide for 10 - 12 hours as rotating organ cultures. The gross appearance of these acrylamide exposed cortices indicated a toxic response compared to the corresponding control cortices cultured for the same period in serum-free medium. The acrylamide exposed cortices were beginning to fragment, and there was a slight turbidity due to the presence of free cells in the culture medium. The acrylamide exposed cortices were trypsin dissociated, and the aggregates formed by these cells in serum-free control medium were monitored after 24 hours of aggregate culture. This acrylamide exposure prior to tissue dissociation did

affect the appearance of the 24 hour aggregates. These aggregates were smaller than their corresponding control aggregates and not quite as compact. However, their appearance was strickingly improved over freshly dissociated cortex cells aggregated 24 hours in the continuous presence of 10 mM acrylamide. The aggregation of the control cultures was not affected by the 10 - 12 hours prior organ culture exposure in serum-free culture medium.

The aggregation of pre-exposed (10 - 12 hours in 10 mM acrylamide as organ cultures) was monitored at time points prior to 24 hours. The first stages (first 2 hours) were similar to the control situation. By 3 hours and continuing through 6 -7 hours, the acrylamide pre-exposed cells remained small loose cluster aggregates with some secondary fusions, while the control cultures continued to grow and began to become compact. By 12 hours of culture, however, the acrylamide pre-exposed cultures appeared to be recovering from this toxic effect, and began to compact. This apparent recovery continued to the 24 hour termination time point.

The organ culture pre-response manipulation was exploited to determine the minimum pre-exposure time to acrylamide required to elicit an effect upon subsequent aggregation of the dissociated cells in serum-free control medium. Thus, corticeswere pre-exposed to 10 mM acrylamide for 2, 5, or 8 hours, and then processed for aggregation. The resultant 24 hour aggregates demonstrated a graded response to the prior acrylamide exposure, ranging from no effect upon aggregation with the 2 hour pre-exposure to a moderate effect after 5 hours pre-exposure to a definite effect following the 8 hour pre-exposure.

Effect of N-Methyl Acrylamide on C x C^{10} Cell Aggregates:

Freshly dissociated C x C^{10} cells were aggregated 24 hours in the presence of 20 mM N-methylacrylamide. The resultant 24 hour aggregate demon-

strated a slightly affected appearance. They were somewhat smaller (reminiscent of 5 mM acrylamide) and they were beginning to show signs of the more jagged aggregate contours characteristic of 8 and 10 mM acrylamide exposure. The aggregation of C x C^{10} cells in the presence of 20 mM N-methyl acrylamide was followed at early stages of aggregation (2, 3, 6, 7, 8, and 9 hours). There was no appreciable difference in appearance of these aggregates to corresponding controls in serum-free medium. Apparently, the slight inhibitory effect of N-methyl acrylamide on 24 hour aggregation begins later in the aggregation process, and thus has a different type of kinetics than 10 mM acrylamide inhibition.

Cortices were pre-exposed to 20 mM N-methyl acrylamide as organ cultures for 12 hours, and then dissociated and aggregated in control medium. The pre-exposed cells did demonstrate some noticeable differences from controls during the time points from 3 to 8 hours of aggregation. Similar to acrylamide, these aggregates were loose, flat and had less regular contours than control aggregates. However, these aggregates seemed to undergo more early secondary fusions causing these early aggregate clusters to be larger than the corresponding controls. While still somewhat larger and looser, these pre-exposed aggregates began to become compact by 9 - 11 hours of aggregation, and by the 24 hour termination point they looked similar to the controls.

Acetylcholinesterase Levels in C x C^{10} Cultures Exposed to Acrylamide and N-methylacrylamide:

Acetylcholinesterase (AchE) levels were assayed both in aggregation cultures and in intact C x C^{10} cortices maintained as organ cultures. AchE levels were monitored at 2 time points following exposure to acrylamide: 8 hours and 24 hours. AchE specific activities are expressed both in terms of enzyme rate per mg of protein and enzyme rate per cortex used (cortex equiva-

lent in the case of aggregates). In light of the cummulative error resulting from the vagaries of tissue dissection, dispensing equal numbers of cells in the case of aggregate studies, and the biochemical assays, I submit that percentage differences between control and experimental cultures that are less than ≈10% are probably of questionable significance.

Table I indicates the mg/protein/cortex, nmoles/min/cortex (cortex equivalent), and nmoles/min.mg protein AchE specific activities for non-cultured 10-day embryonic chick cortices. These indices for the non-cultured cortices represent the baseline values against which percentage changes are calculated for the other experimental cultures listed in this table.

C x C^{10} cortices maintained as organ cultures for 8 hours in control medium demonstrate a somewhat reduced mg/cortex/ content (18%↓ in response to the culture exposure in serum-free medium. A significant proportion of this reduced protein content represents generalized tissue degeneration as AchE specific activity in terms of nmoles/min/cortex is reduced ≈11%. However, the effects of the culture conditions are somewhat more pronounced in reducing the total protein content than the relative amount of AchE as seen in the slight increase in AchE specific activity expressed in terms of nmoles/min/mg protein. It should be noted that the level of tissue decay represented by this protein decline is not sufficient to affect the size or morphology of 24 hour aggregates formed when these pre-incubated cortici are dissociated and the cells are reaggregated.

Exposure of the 8 hour organ cultures to 10 mM acrylamide caused a marked decrease in protein content (3%↓). This reduced protein content is <u>not</u> reflected proportionately in the levels of AchE activity. The nmoles/min/cortex value is down only 8% indicating that the depressed protein content has

not yet led to generalized degeneration. AchE specific activity expressed in terms of mg protein demonstrates a 45% increase, indicating that the AchE enzyme is not a target of the reduced protein levels in response to acrylamide.

Table II examines the same data for acrylamide exposure, however, now expressed relative to its corresponding control culture rather than to the non-cultured cortex starting material. Expressed this way, there is no significant change in AchE enzyme activity per cortex. The AchE activity per mg protein is elevated (28%) in proportion to the reduced protein content (24%). Indicating again, that the toxic effect of 10 mM acrylamide on total cellular protein does not encompass a corresponding decline in AchE levels; and certainly not a specific inhibition of AchE activity.

When the control organ cultures are maintained in serum-free medium for 24 hours, Table I again indicates that such culture conditions cause a decrease in total protein content (28%↓). This reduced protein content probably represents generalized tissue and cell decay as corroborated by the proportionate decline (21%) in AchE activity per cortex used. Correspondingly, the AchE activity per mg protein remains essentially unchanged.

$C \times C^{10}$ cortices maintained in serum-free medium containing 10 mM acrylamide exhibited a massive decrease in protein content (75%↓). A considerable portion of this protein decrease was translated into tissue distruction as evidenced by the 49% decline in AchE activity per cortex used. This correlates well with the poor gross appearance of these cultures (fragmented, loose cortices with free cells in the medium). While there was considerable tissue damage, the loss of AchE activity did not parallel the large magnitude of the protein decrease. This is similar to the effect of acrylamide on 8 hour cultures. As a result, the nmoles/min/mg protein specific

activity rose precipitously. When the cultures of 24 hour cortices in 10 mM acrylamide are compared to their corresponding control (Table II), there was a 68% drop in protein content/cortex, corresponding to a 36% decline in AchE activity per cortex used. Again, the loss of AchE activity did not keep pace with protein loss yielding a large increase in AchE activity per mg protein, further evidence that AchE is not a specific target for acrylamide inhibition in this system.

When C x C^{10} cortices are trypsin dissociated and aggregated for 8 hours in serum-free medium, there is a large decrease in relative protein content (57%↓) compared to the equivalent amount of initial cortex material in situ. This is not unreasonable considering the drastic events of neural tissue dissociation. There was a proportionate 55% decline in AchE activity per cortex equivalent corresponding to this dissociation-related tissue damage.

Aggregation of the dissociated cells for 8 hours in 10 mM acrylamide again caused an enhanced decrease in protein control per cortex equivalent (73%↓). Table I also indicates that this additional protein loss was not yet translated into additional gross tissue destruction; i.e., AchE-activity is down only slightly more than 8 hour control cultures. Similar to the case with cortex organ cultures, the acrylamide induced decline in protein content was not paralleled by a proportioanl decrease in AchE activity. Thus, the 60% increase in AchE activity per mg protein.

When compared to their 8 hour control aggregates (Table II), we again see the acrylamide induced toxicity on protein content (38%↓) which is not correlated with a corresponding decline in Ache activity (i.e., AchE activity per mg protein is up the proportionate 34% and AchE activity per cortex equivalent remain relatively unchanged).

When control aggregates are maintained in serum-free medium for 24 hours, the number do not change dramatically from the situation in 8 hour control aggregate cultures. There is a slight drop in AchE specific activation, which probably corresponds to the additional culture time in the minimal serum-free medium.

Aggregation for 24 hours in the presence of 10 mM acrylamide exacerbates this general decline. Protein content is again dramatically decreased by the acrylamide toxicity (89% compared to the original starting tissue and 66% compared to the corresponding 24 hour control aggregates). The aggregate tissue atrophy seen in the gross morphology of the aggregates is again correlated with the decline in AchE activity per cortex equivalent compared to the control aggregates. However, AchE again does not appear to be the primary target of acrylamide toxicity for the AchE activity per mg protein is actually 44% higher than the corresponding control. Again, this reflects the fact that the protein content is going down faster in response to acrylamide than tissue destruction is eliminating AchE.

Like acrylamide, N-methyl acrylamide is toxic and does not appear to act directly upon AchE. The N-methyl acrylamide is of course less toxic, with twice the dosage having a less dramatic and more evenhanded general toxic effect upon the cultures.

Finally, there is the direct evidence that acrylamide does not specifically inhibit AchE activity. When acrylamide was added to a final concentration of 100 mM to the reaction mixture of AchE assay for non-cultured cortices, it did <u>not</u> change the slope of the rate reaction curve.

Effect of Acrylamide, N-Methylacrylamide and Crotonamide on Viability and Plating Efficiency of NIE-115 Cells:

The neuroblastoma cell line NIE-115 was studied in a proliferating, non-neuronal stage and after differentiation by cAMP into a neuron-like stage of differentiation.

The LD_{50} dose for proliferating cells, as determined by trypan blue exclusion, was 2.3 mM for acrylamide (AA) and 50 and 75 mM for N-methylacrylamide (NMA) and crotonamide (CA), respectively. For differentiated cells the LD_{50} doses were 1.0 (AA), 35 (NMA), and 50 mM (CA).

The ID_{50} doses for plating efficiency of proliferating neuroblastoma cells were 1.0 (AA), 25 (NMA), and 50 mM (CA) while the ID_{50} doses for cell proliferation were 2.0 (AA), 20 (NMA) and 50 mM (CA).

Hence, it can be seen that the toxicity of the three acrylamides parallel the reported in vivo ranking, with plating efficiency being more sensitive to inhibition than cell proliferation or cell viability. Furthermore, the differentiated cells were more sensitive to each of the acrylamides than the undifferentiated cells. The linearity of the dose-response curves is typified by that for plating efficiency shown in Fig. 1.

Effect of AA, NMA, and CA on Macromolecular Synthesis:

The effect of AA, NMA, and CA on protein, DNA, and RNA content of differentiated NIE-115 cells and log phase cells, is shown in Tables 3 and 4 while Table 5 shows the effect of these chemicals on macromolecular synthesis rates. Low doses of AA in the range of 0.01 mM to 1.0 mM had no effect on protein and RNA content, but did reduce the DNA content, when all cells (trypan blue positive and negative) are included in the analysis and calculation. If one takes into account only the viable cells, then there was a

slight increase in the content of macromolecules as the dose of AA increased. N-methylacrylamide at dose of 10 to 40 mM caused an increase in protein content and in RNA content, while also inducing a decline in the DNA content. These increases were in the range of 10-25%. CA caused similar effects at slightly higher doses (from 30 to 50 mM). These effects induced by NMA and CA are consistent with the morphological differenttiation observed when similar doses were administered to log phase cultures in proliferation studies.

Control 8 day differentiated cells had 713 µg protein, 202 µg RNA, and 4.6 µg DNA per 10^6 cells. This was a 27% increase over log phase cells in the amount of protein and a 13% increase in the RNA content. At the same time the DNA content of the cells dropped by 44%. Differentiated NIE-115 neuroblastoma cells were not affected by the xenobiotics except at doses which caused greater than 50% lethality. Then, only the protein levels were lowered by about 20% (Table 3).

Tables 4 and 5 list the protein, RNA, and DNA synthesis data for log phase and differentiated cells, respectively. Log phase NIE-115 neuroblastoma cells synthesized protein at a rate of 54% higher than that of differentiated cells. Log phase cells also had a 160% higher RNA synthesis rate and a 1790% higher DNA synthesis rate, than did differentiated cells.

Log phase cultures treated with AA did not show any inhibition of macromolecular synthesis until doses which caused greater than 50% lethality were administered. Doses of 2.0 mM AA reduced the protein, RNA, and DNA synthesis rates by only 27%, 27% and 47%, respectively. Both NMA and CA treated cultures did not show any inhibitory effects on protein and RNA synthesis, at doses below 40 mM and 75 mM, respectively. N-methylacrylamide, at dose of 5.0, 10, and 20 mM caused increases in the protein and RNA synthesis rates of log

phase cells, while causing a simultaneous decrease in the DNA synthesis rate. This effect was also seen with doses of CA ranging from 20 to 50 mM. These observations are consistent with the morphological differentiation and reduced growth rates noted earlier, which suggest that moderate doses of NMA and CA induce log phase neuroblastoma (NIE-115) cells to cease proliferating and to begin differentiating.

The protein, RNA, and DNA synthesis rates of differentiated NIE-115 neuroblastoma cells were slightly inhibited by doses which were below the LD_{50} dose (1.0 mM AA, 35 mM NMA, and 50 mM CA). At doses of 1.0 mM AA the protein synthesis rate was reduced by only 12% and RNA synthesis was reduced by 25%. N-methylacrylamide treated cultures showed a 49% reduction in RNA synthesis at 30 mM concentration but only a 12% reduction in the protein synthesis. Crotonamide, at doses of 40 mM inhibited protein synthesis by 11% and RNA synthesis by 39%.

Effect of Acrylamides on Acetylcholinesterase (AchE) activity:

Undifferentiated log phase neuroblastoma cells express small amounts of AchE activity (Augusti-Tocco and Sato, 1969; Anano et al, 1972). Upon differentiation as much as 20 to 30-fold increase is sometimes noted (Blume et al, 1970; Schubert et al, 1971; Kates et al, 1961; Harkins et al, 1972; Prasad and Vernadakis, 1972). In the research reported herein a ten-fold increase (82.1 nmol product formed/min/mg protein to 865 nmol product formed/min/mg protein) in AchE activity was observed when log phase cultures were induced to differentiate with serum-free medium and 0.5 mM dibutyryl cAMP.

Non-differentiated and differentiated cultures were treated with various doses of AA, NMA, and CA under standardized conditions. Acetylcholinesterase activity was determined by the colorimetric procedure of Ellman et al (1961).

When measuring AchE activity it is important to block the action of other esterases which may be present and may interfere with the reaction. There are other esterases present in neuroblastoma cells. Butyrylesterase is not present in significant amounts in cells of neuronal origin (Schubert et al, 1971); Prasad and Vernadakis, 1972), but cholinesterase and acetylesterase are present in measurable amounts in neuroblastoma cells (Blume et al, 1970). These pseudoesterases are selectively inhibited by iso-OMPA (El-Badawi and Schenk, 1967; Blume et al, 1970), which was added to all reaction mixtures. Controls run with Eel electric organ AchE, in the presence of iso-OMPA indicated only a 5% decrease in AchE activity.

Log phase cultures treated with AA showed a substantial decrease in AchE activity at concentrations above 0.5 mM. Control AchE activity in log phase cells was 82.1 nmol product formed/min/mg protein. The LD_{50} by AA for AchE activity in log phase cells was 2.0 mM (37 nmol product/min/mg protein), with linear portion of the dose response curve showing a slope of -13 between doses of 0.5 mM AA and 4.0 mM AA.

Log phase cultures of NIE-115 neuroblastoma cells treated with NMA at doses of 10 to 30 mM were induced to increase their levels at AchE activity by as much as 56%, from 82.1 nmol of product formed/min/mg protein to 128 nmol/min/mg protein. Similar results were observed with 30 to 50 mM doses of CA (Fig. 2). Higher doses of both NMA and CA led to inhibition of AchE activity. N-methylacrylamide treated cultures had a dose response curve with a slope of -1.5, as compared to -13 for AA, in the linear response region from 30 to 100. The ID_{50} for NMA was measured at a dose of 75 mM. Crotonamide-treated cultures showed a linear dose response from 40 to 100 mM with a slope of -1.4, which was similar to that measured for NMA, but much less than that

observed for AA. The ID_{50} for CA dosed cultures was at 100mM.

Differentiated cells were more sensitive to the effects of the xenobiotics, and had steeper dose response curves (Fig. 3). The dose response curve for AA was very steep from 0.01mM or 0.5mM, with a slope of -100. The dose response curve for AA became less steep from 0.5 to 5.0mM with a slope of -8.3. The ID_{50} for AChE activity in AA treated differentiated cells was at a dose of 0.5mM as compared to 2.0mM for log phase cells. N-methylacrylamide-treated differentiated cells had an ID_{50} at 35mM with a slope of -1.6, in contrast to CA-dosed cells which had an ID_{50} or 60mM and a slope of -0.59.

Neuron Specific Enolase (NSE) and Acrylamide Toxicity:

Recent reports implicate NSE as the possible site for AA neurotoxicity (Howland et al, 1980a). Hence, the study was expanded to include the effect of acrylamides on neuron specific enolase (NSE) and the non-neuronal end-points, survival and proliferation. Forty-eight hour survival and proliferation of the cells showed a graded dose response. A concentration of 0.01mM reduced proliferation rate by 10% while 5mM was completely inhibitory. When survival was used as an endpoint, the differentiated cells showed increased sensitivity; a dose of 3mM for 48 hrs was lethal to the culture. Exposure to 0.5mM for 48 hrs resulted in a 50% reduction in the activity of neuron-specific enolase (from 0.8 umol to 0.4 umol product formed/min/mg protein) while the activity of non-neuron specific enolase was unaffected. Furthermore, these doses did not affect AChE activity, suggesting some specificity of acrylamide toxicity.

N-methylacrylamide inhibited NSE activity to a lesser extent than AA. The ID_{50} dose was 50mM NMA. A shoulder was evident from 1.0 to 10 mM NMA with the linear part of the dose response curve extending from 10 to 60 mM NMA. This linear region had a slope of -1.5.

Crotonamide also was less reactive in inhibiting the activity of NSE. One hundred and sixty-fold higher doses of CA were necessary to reach the ID_{50} (80 mM). The slope of the CA dose response curve was -0.66 in the linear response region from doses of 20 to 100mM. This slope was only half that measured for NMA. A shoulder was noted from doses of 1.0 to 20 mM CA.

The total enolase activity of differentiated N1E-115 neuroblastoma cells was not greatly affected by either AA, NMA, or CA.

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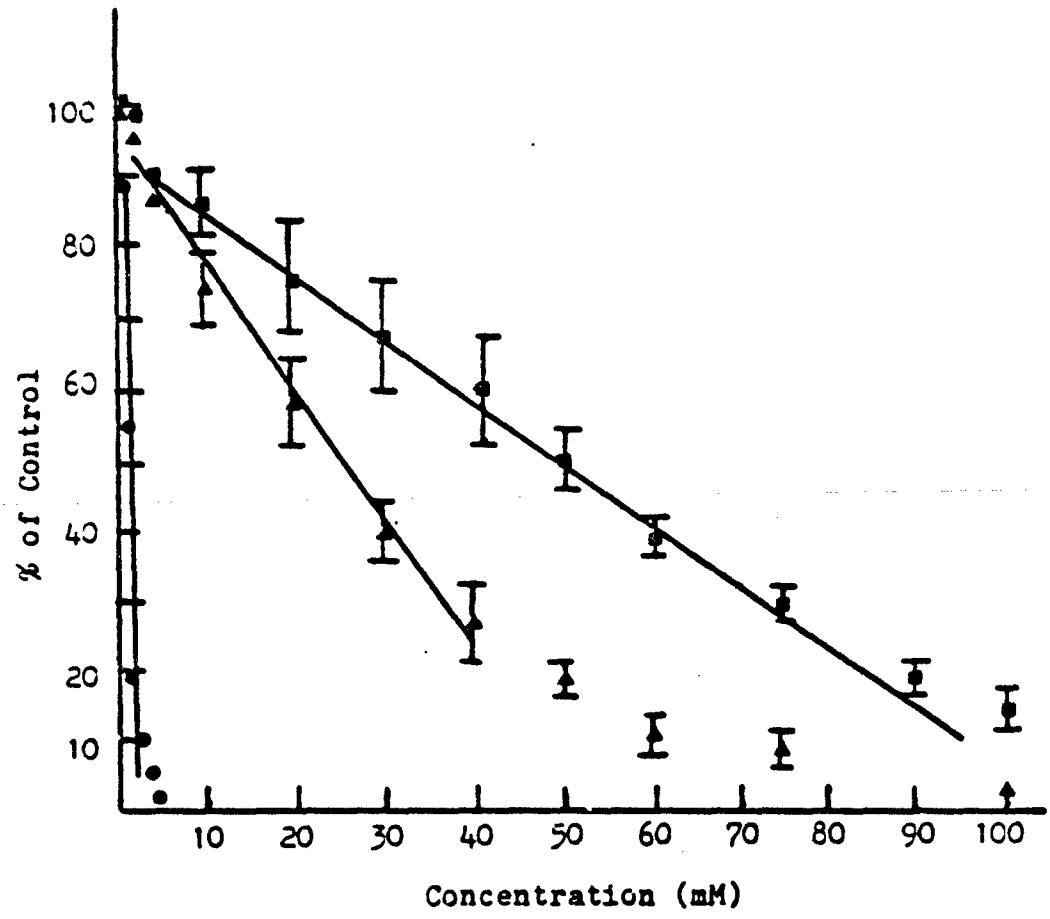


Figure 1 The effect of 48 hr exposure to acrylamide (●——●), N-methylacrylamide (▲——▲), or crotonamide (■——■) on the plating efficiency of log phase N1E-115 neuroblastoma cells (± S.E.).

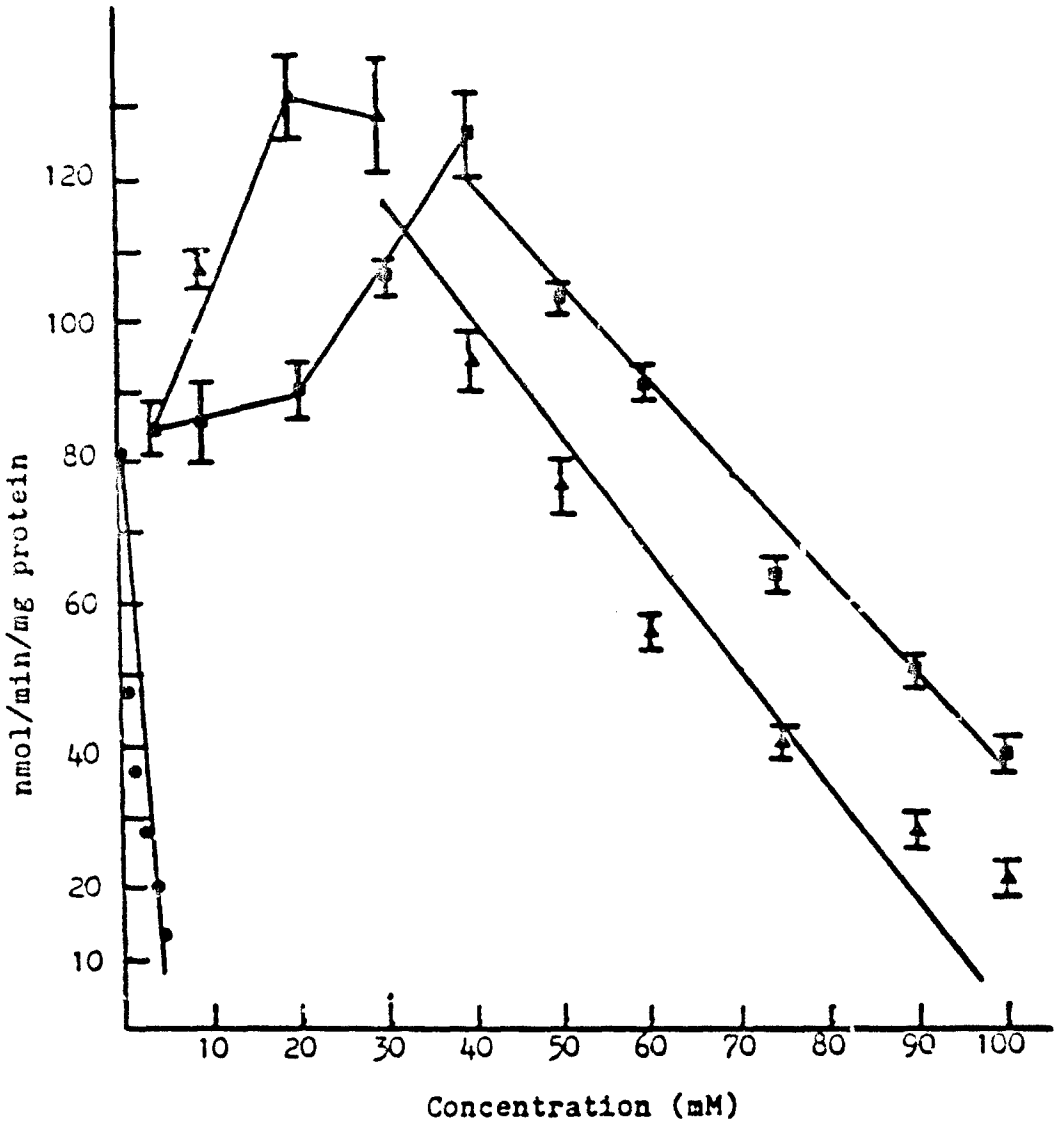


Figure 2 The effect of 48 hr exposure to acrylamide (●——●), N-methylacrylamide (▲——▲), or crotonamide (■——■) on the acetylcholinesterase activity of log phase N1E-115 neuroblastoma cells (± S.E.).

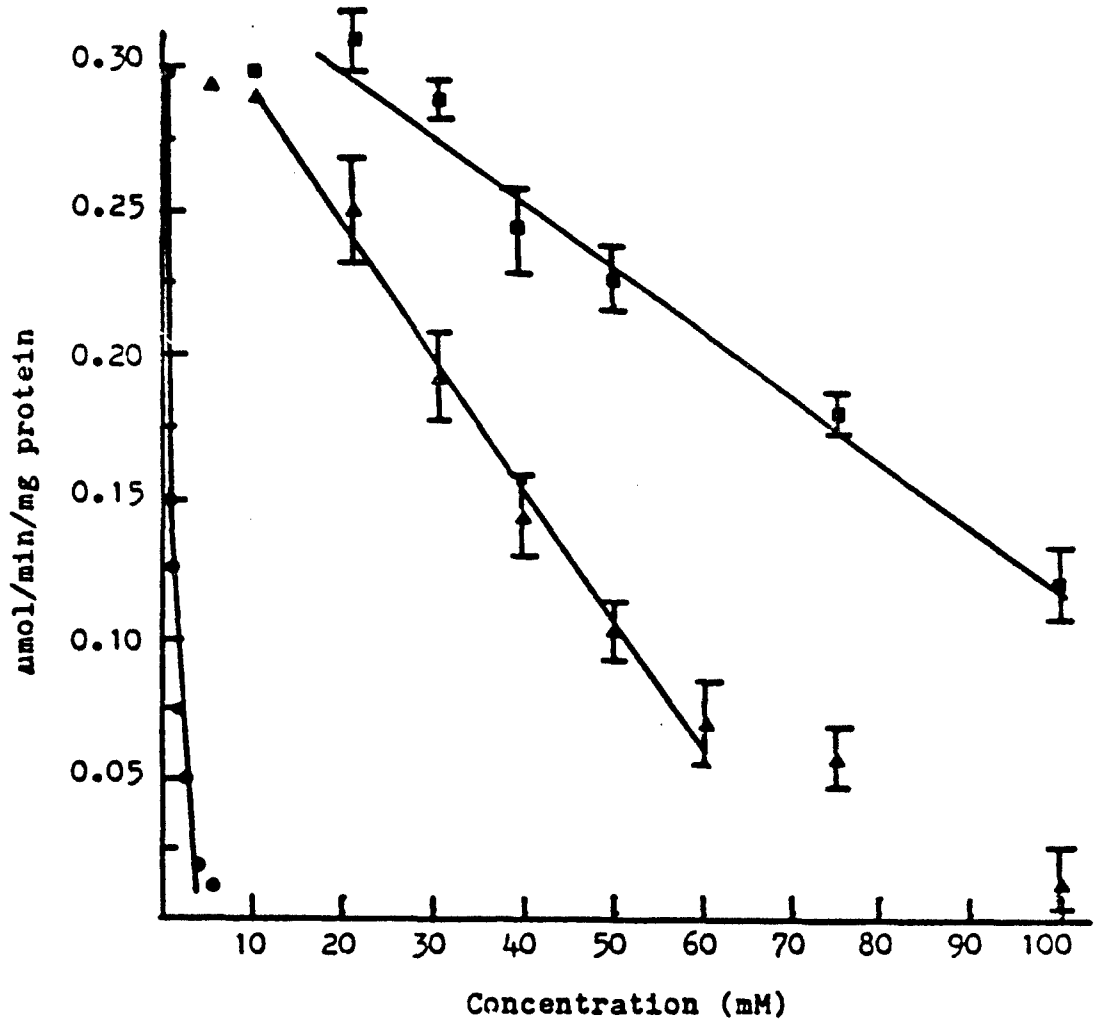


Figure 3 The effect of 48 hr exposure to acrylamide (●——●), N-methylacrylamide (▲——▲), or crotonamide (■——■) on the neuron specific enolase activity of differentiated N1E-115 neuroblastoma cells (± S.E.).

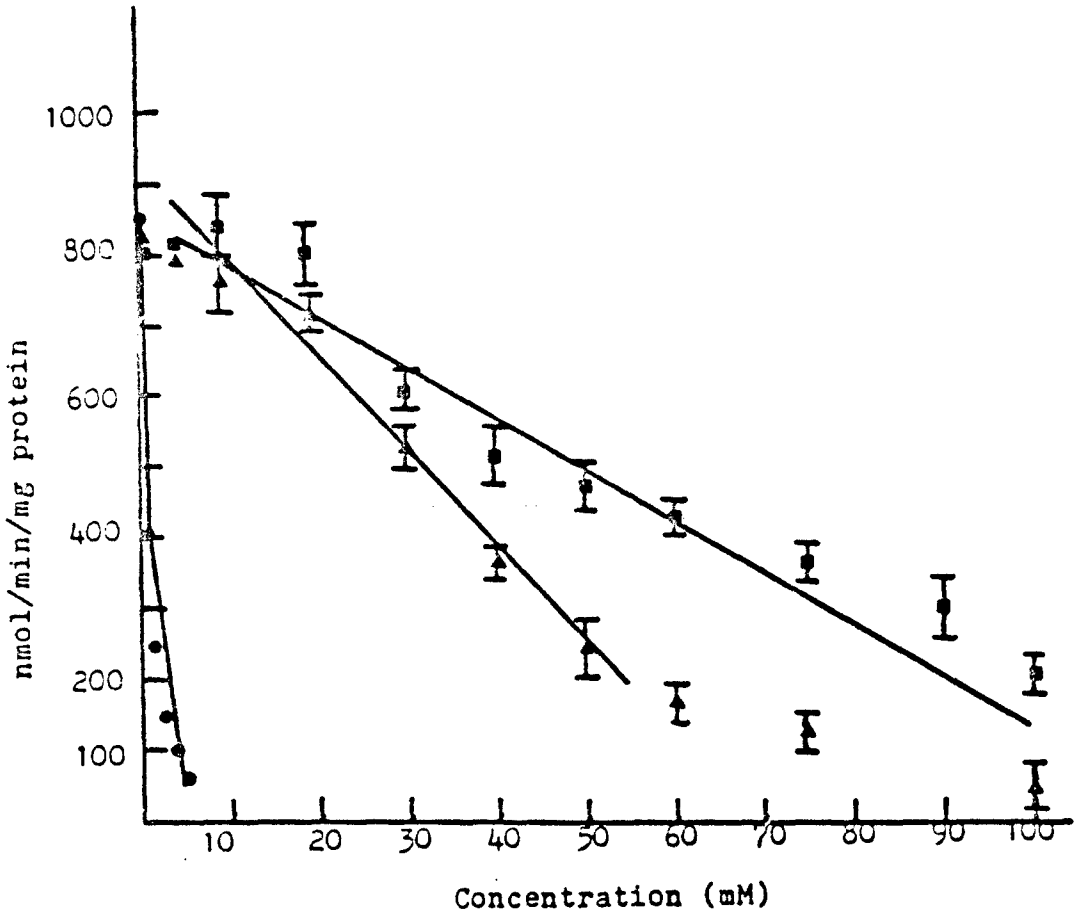


Figure 4 The effect of 48 hr exposure to acrylamide (●——●), N-methylacrylamide (▲——▲), or crotonamide (■——■) on the acetylcholinesterase activity of differentiated N1E-115 neuroblastoma cells (± S.E.).

Table 4 Effect of acrylamide on protein content and acetylcholinesterase activity by chick brain organ culture.

Specimen	Mg protein/cortex (equivalent)	nmoles/min/cortex (equivalent)	nmoles/min/mg protein
Non-cultured Cortices	0.097 (100%)	5.3 (100%)	54.1 (100%)
8 hr Control Cortices	18%↓	11%↓	12%↑
8 hr Cortices in 10 mM Acrylamide	37%↓	8%↓	45%↑
24 hr Cont Cortices	24%↓	21%↓	4%↑
24 hr Cortices in 10 mM Acrylamide	75%↓	49%↓	132%↑
8 hr Control Aggregates	57%↓	55%↓	20%↑
8 hr Aggregates in 10 mM Acrylamide	73%↓	58%↓	60%↑
24 hr Control Aggregates	60%↓	64%↓	10%↓
24 hr Aggregates in 10 mM Acrylamide	84%↓	84%↓	29%↑

Table 5 Effect of acrylamide and n-methylacrylamide on protein content and acetylcholinesterase activity of aggregates of brain cells.

Treated Culture Compared to Its Respective Control	Mg Protein / Cortex (equiv.)	nmoles/min/cortex (equiv.)	nmoles/min/mg/protein
8 hr Cortices in 10 mM Acrylamide	24%↓	4%↑	28%↑
24 hr Cortices in 10 mM Acrylamide	68%↓	36%↓	124%↑
8 hr Aggregation in 10 mM Acrylamide	38%↓	6%↓	34%↑
24 hr Aggregation in 10 mM Acrylamide	66%↓	47%↓	44%↑
24 hr cortices in 20 mM N-methyl acrylamide	32%↓	37%↓	9%↓
24 hr Aggregation in 20 mM N-methyl acrylamide	34%↓	34%↓	4%↓

TABLE

THE EFFECT OF 48 HOUR ACRYLAMIDE (AA), N-METHYLACRYLAMIDE (NMA), OR CROTONAMIDE (CA) EXPOSURE ON THE MACROMOLECULAR CONTENT OF DIFFERENTIATED N1E-115 NEUROBLASTOMA CELLS

Xenobiotic	Dose (mM)	µg Protein*		µg RNA*		µg DNA*	
		10^6 cells	10^6 VC**	10^6 cells	10^6 VC**	10^6 cells	10^6 VC**
Control	-	713 ± 15	713 ± 15	202 ± 6.4	202 ± 6.4	4.6 ± 0.3	4.6 ± 0.3
AA	0.01	708 ± 23	761 ± 25	193 ± 12	208 ± 13	4.9 ± 0.2	5.2 ± 0.2
AA	0.05	698 ± 21	793 ± 26	213 ± 10	242 ± 12	4.8 ± 0.3	5.5 ± 0.3
AA	0.10	718 ± 16	835 ± 22	198 ± 14	230 ± 17	4.8 ± 0.2	5.6 ± 0.3
AA	0.50	683 ± 20	1030 ± 30	213 ± 11	323 ± 16	5.3 ± 0.4	8.0 ± 0.6
AA	1.00	646 ± 26	1240 ± 50	191 ± 11	367 ± 19	4.1 ± 0.3	7.9 ± 0.6
AA	2.00	588 ± 17	2560 ± 67	194 ± 13	843 ± 40	5.4 ± 0.4	24 ± 2
NMA	5.0	718 ± 17	772 ± 19	220 ± 8.0	237 ± 9.2	4.9 ± 0.3	5.3 ± 0.4
NMA	10.0	711 ± 20	808 ± 22	205 ± 13	233 ± 14	5.0 ± 0.3	5.7 ± 0.4
NMA	20.0	681 ± 18	873 ± 22	200 ± 12	256 ± 15	4.9 ± 0.2	6.3 ± 0.3
NMA	30.0	677 ± 23	1170 ± 45	219 ± 7.9	378 ± 14	5.2 ± 0.3	9.0 ± 0.5
NMA	40.0	611 ± 19	1490 ± 40	217 ± 16	529 ± 36	5.6 ± 0.3	14 ± 0.7
NMA	50.0	303 ± 16	1317 ± 46	181 ± 8.9	787 ± 35	5.2 ± 0.3	23 ± 2
CA	5.0	691 ± 20	691 ± 20	173 ± 10	173 ± 10	4.1 ± 0.3	4.1 ± 0.3
CA	10.0	708 ± 18	730 ± 19	236 ± 16	242 ± 17	5.0 ± 0.3	5.2 ± 0.3
CA	20.0	701 ± 19	754 ± 21	188 ± 17	202 ± 18	4.9 ± 0.4	5.3 ± 0.4
CA	30.0	684 ± 22	834 ± 25	208 ± 16	254 ± 18	5.4 ± 0.3	6.6 ± 0.4
CA	40.0	667 ± 20	981 ± 30	196 ± 6.5	288 ± 10	5.4 ± 0.2	8.0 ± 0.3
CA	50.0	602 ± 24	1180 ± 47	178 ± 12	349 ± 24	5.4 ± 0.4	11 ± 0.8
CA	75.0	387 ± 16	992 ± 36	186 ± 10	477 ± 28	4.9 ± 0.2	12 ± 0.7

*± S.E.
**Viable Cells

TABLE 7

THE EFFECT OF 48HOUR ACRYLAMIDE (AA), N-METHYLACRYLAMIDE (NMA), OR CROTONAMIDE (CA) EXPOSURE ON THE MACROMOLECULAR SYNTHESIS RATES OF LOG PHASE N1E-115 NEUROBLASTOMA CELLS

Xenobiotic	Dose (mM)	CPM* / µg Protein	CPM* / µg RNA	CPM* / µg DNA
Control	-	117 ± 11	2160 ± 50.8	2480 ± 101
AA	0.05	105 ± 15	1730 ± 97.0	2310 ± 98.5
AA	0.10	113 ± 16	2310 ± 114	2060 ± 76.9
AA	0.50	117 ± 22	1830 ± 97.5	1810 ± 112
AA	1.00	100 ± 13	1820 ± 121	2130 ± 95.9
AA	2.00	85.3 ± 18	1580 ± 79.3	1320 ± 89.3
AA	3.00	57.5 ± 17	859 ± 66	66.8 ± 15
NMA	5.0	341 ± 32	2200 ± 107	1910 ± 99.3
NMA	10.0	376 ± 23	2430 ± 135	1200 ± 77.2
NMA	20.0	276 ± 15	2680 ± 85.9	976 ± 56
NMA	30.0	231 ± 18	2110 ± 117	87.9 ± 12
NMA	40.0	153 ± 7.4	1010 ± 66.4	42.3 ± 4.8
NMA	50.0	70.2 ± 3.2	496 ± 12	70.7 ± 5.8
CA	10.0	133 ± 22	1960 ± 114	2320 ± 114
CA	20.0	173 ± 19	2280 ± 125	1990 ± 142
CA	30.0	236 ± 30	2670 ± 111	1770 ± 130
CA	40.0	244 ± 10	2430 ± 88.4	1350 ± 77.3
CA	50.0	203 ± 12	1840 ± 73.7	863 ± 23
CA	75.0	106 ± 7.4	321 ± 22	66.8 ± 7.5

*± S.E.

TABLE 8

THE EFFECT OF 48 HOUR ACRYLAMIDE (AA), N-METHYLACRYLAMIDE (NMA), OR CROTONAMIDE (CA) EXPOSURE ON THE MACROMOLECULAR SYNTHESIS RATES OF DIFFERENTIATED N1E-115 NEUROBLASTOMA CELLS

Xenobiotic	Dose (mM)	CPM* / µg Protein	CPM* / µg RNA	CPM* / µg DNA
Control	-	76.0 ± 7.3	828 ± 23	131 ± 14
AA	0.01	82.7 ± 2.5	886 ± 22	183 ± 14
AA	0.05	80.6 ± 5.6	751 ± 15	112 ± 10
AA	0.10	79.6 ± 3.5	689 ± 15	197 ± 11
AA	0.50	82.9 ± 5.4	677 ± 12	155 ± 15
AA	1.00	69.0 ± 0.75	625 ± 9.6	146 ± 14
AA	2.00	67.1 ± 2.6	609 ± 10	53.3 ± 8.5
NMA	5.0	89.4 ± 3.2	1170 ± 16	104 ± 4.3
NMA	10.0	87.5 ± 6.0	1100 ± 40	89.1 ± 5.4
NMA	20.0	82.8 ± 4.8	814 ± 23	44.0 ± 0.42
NMA	30.0	78.3 ± 9.2	601 ± 11	58.7 ± 0.69
NMA	40.0	62.1 ± 0.92	312 ± 4.9	0
NMA	50.0	33.8 ± 1.1	7.02 ± 0.33	0
CA	5.0	88.2 ± 4.9	715 ± 18	82.2 ± 8.2
CA	10.0	79.3 ± 3.2	730 ± 10	136 ± 7.4
CA	20.0	81.3 ± 2.2	619 ± 13	122 ± 2.4
CA	30.0	74.7 ± 2.9	820 ± 7.5	117 ± 10
CA	40.0	78.3 ± 3.6	433 ± 5.9	66.1 ± 0.55
CA	50.0	66.3 ± 0.94	265 ± 2.6	73.2 ± 1.2
CA	75.0	44.7 ± 1.3	97.8 ± 0.83	49.7 ± 2.3

* ± S.E.

3. An In Vitro Alternative for Testing the Effect of Organophosphates on Neurotoxic Esterase Activity

3.1 INTRODUCTION

The organophosphorus (OP) compounds represent a diverse group of chemicals widely used as pesticides, lubricants, hydraulic fluids, plasticizers, and flame retardents (1). Over 200 million pounds of OP biocidal compounds are produced annually world-wide (2). In view of the ubiquitous nature of these compounds, it is essential to health and safety that the toxicity of these compounds be anticipated and understood prior to full scale production of new OPs. Hence, the importance of appropriate methods for screening and mechanism studies.

There have been many outbreaks of organophosphorus ester-induced delayed neurotoxicity (OPIDN) recorded in recent history affecting man and other non-target organisms. Abou-Donia (3) reported that 40,000 cases of delayed neurotoxicity in man have been documented. The first cases fof OPIDN were reported to have occurred at the end of the nineteenth century in tuberculosis patients treated with phospho-creosote. This is a mixture of uncharacterized esters derived from coal tar phenols and phosphoric acid (4). In 1930, a massive outbreak of OPIDN affecting up to 20,000 persons occurred as the result of the ingestion of an illegal liquor substitute, known as Jamaica ginger, which contained triortho cresyl phosphate (TOCP). Many other outbreaks of OPIDN have been recorded since 1930, the most recent of which occured in 1975 and affected scores of Egyptian farmers and domestic livestock (5). The OP insecticide leptophos, also known as phosvel, which was unregistered in the

United States, was the toxic agent. In 1976, 500 Pakistani workers were acutely poisoned by mishandling the OP insecticide malathion; five fatalities resulted (5). The World Health Organization (WHO) reported that in 1972, 500,000 persons were poisoned by pesticides exported to third world nations in large quantities. These pesticides often are applied excessively and improperly by untrained or illiterate workers (5).

The OP compounds produce two distinct and unrelated types of toxicity. The first is an acute toxicity which occurs within minutes following exposure. It is characterized by excessive cholinergic stimulation. This results from the binding of an OP to acetylcholinesterase (AChE) and subsequent inactivation of this neuronal enzyme. Without active AChE present at post synaptic membrane surfaces, the neurotransmitter acetylcholine is not degraded; thus it accumulates. This leads to excessive stimulation of the muscarinic and nicotinic receptors within the nervous system. Some symptoms include excessive lacrimation, salivation, sweating, involuntary muscle contraction, urination, defecation, and weakness. Death usually results from asphyxiation due to excessive bronchoconstriction, bronchosecretion, and paralysis of the respiratory muscles (6). The mechanism of this type of toxicity is well understood. High risk populations are monitored for exposure by assaying RBC and plasma cholin-esterase, a decrease being indicative of exposure.

The second type of toxicity associated with these compounds is organophosphorus ester-induced delayed neurotoxicity (OPIDN) (3). It is characterized clinically by ataxia and paralysis beginning in the distal portion of the hind limb and advancing proxi-

mally. The forelimbs become involved in severe cases. There is a latent period of 6 to 14 days between exposure to the OP and onset of clinical symptoms (3). There is a wide species variation in sensitivity to OPIDN, with man, chickens, and cats being most sensitive, while rodents are least sensitive (3). In addition, children and young animals are much less sensitive to OPIDN than are adults and mature animals. Recovery from OPIDN is extremly slow and often incomplete (1).

The histopathology of OPIDN has been extensively investigated by Bouldin and Cavanagh (7,8). In 1968, Johnson (9) found that a neurotoxic dose of ^{32}p-labeled diisopropyl fluorophosphate (DFP), labeled hen brain homogenates in vitro. Preincubation of the homogenate with a non-neurotoxic OP reduced labeling by the ^{32}p-DFP. Preincubation with a neurotoxic OP led to further reduction in labeling by ^{32}p-DFP. Johnson proposed that the fraction of labeling reduced by a neurotoxic OP but not by the non-neurotoxic OP might represent a biochemical target site for the action of neurotoxic OPs. In 1970, Johnson (10), showed a positive correlation between phosphorylation of this brain fraction and clinical development of OPIDN in hens. This fraction which was found capable of hydrolyzing the ester phenyl phenylacetate, was subsequently named neurotoxic esterase (NTE). Certain carbamates and sulphonates, in addition to phosphates, were found to bind to and inhibit NTE, but they did not cause delayed neuropathy. In vivo administration of sulphonates or carbamates actually provided protection to hens against subsequent challenge doses of neurotoxic OPs. This protection lasted until 70% of the NTE again became available for phosphorylation. Simple inactivation of NTE is not responsible for OPIDN, but the chemical nature of the bound compound is crucial. In 1974,

Johnson (11) reported that phosphinates behaved like carbamates and sulphonates against NTE, inhibiting it but not causing OPIDN. It has been proposed that the development of OPIDN requires not only phosphorylation of NTE, but subsequent hydrolysis of a phosphoryl ester bond to leave a charged monosubstituted phosphoric acid group attached to the enzyme. This charged complex is thought to be responsible for disrupting the normal unction of the axon thereby causing neuropathy. Further evidence for this process, known as aging, came in 1979 when the kinetics of NTE aging was studied (12). Aging occurred with a half-life of 2-4 minutes after which reactivation of NTE with potassium fluoride was impossible. The Mechanism of aging of NTE seems to be distinctly different from the better-characterized dealkylation aging of the cholinesterase. Much is still unknown about NTE and OPIDN. The nature and sequence of events between phosphorylation and aging of NTE, and the production of axonal degeneration is completely unknown. The physiological significance of NTE is a mystery as well, since its activity within the neuron does not seem to be essential for cell survival or funtion.

In view of the large number of new OPs produced annually, as well as the potential for neurotoxicity of many other compounds arriving in the market, rapid, inexpensive, predictive toxicity tests are desirable. As part of an overall strategy to develop a battery of in vitro tests for neurotoxicity evaluation and for screening, we have embarked on studies to develop a cell culture test which would be predictive of organophosphorus-induced delayed neurotoxicity. Presently, in vivo testing of OPs with the hen is the method of choice for screening OPs for delayed neurotoxic potential. The hens are dosed with an OP and observed for clinical

signs of OPIDN for several weeks. After this period the hens may be sacrificed and their brains assayed for NTE to yield a more sensitive measure of delayed neurotoxic potential of the OPs in question (13,14). Alternately, OPs may be added directly to brain homogenates from normal hens and then assayed for NTE activity. In order to parallel these two approaches (treatment of whole organisms or treatment of brain homogenates) we have examined the effect of OPs on NTE activity in exposed whole cells of neuroblastoma cultures and on the NTE activity of neuroblastoma cell homogenates, and have compared the toxicity ranking in cell cultures with that reported for the hen brain assay.

3.2 MATERIALS AND METHODS

Clone N1E-115, a subclone of the mouse neuroblastoma clone C-1300 (15), was obtained from M. Nirenberg of NIH, and used as the cellular target. Cell cultures were grown as monolayers in 75 cm^2 polystyrene flasks (Corning Glass Works) with Dulbecco's modification of Eagle's minimum essential medium (DMEM; Flow Labs). The medium was supplemented with newborn bovine serum (Flow Labs) to a concentration of 10% and buffered with 2700 mg/l of sodium bicarbonate. Cultures were maintained in an environment of 10% CO_2-90% air at 37°C. These cells possess the capability to differentiate morphologically, biochemically, and electrophysiologically by manipulating culture conditions appropriately (16). For the experiments reported herein, the cell cultures were differentiated by withdrawal of serum and addition of 0.5mM dibutyryl adenosine 3^1, 5^1-cyclic monophosphate (cAMP; Sigma) when the cells had reached stationary phase. All OP stocks were prepared at a concentration of 20mM in DMSO (Fisher Scientific) and added to whole cultures or homogenates as indicated. The following organophosphate compounds

were obtained from the USEPA Analytical Reference Standards Repository, Research Triangle Park, N.C.: O-methyl O-4-bromo-2, 5-dichlorophenyl phenylphosphonothioate (leptophos), leptophos-oxon, O-ethyl O-4-nitrophenyl phenylphosphonothioate (EPN), octamethyl pyrophosphoramide (OMPA), O, O-diethyl O-p-nitrophenyl phosphate (paraoxon). All reference standards were greater than 98% pure. N, N-diisopropylphosphorodiamidic fluoride (mipafox) was kindly provided by M.B. Abou-Donia, Duke University, as was the substrate, phenyl valerate. DFP was obtained from Sigma.

After exposure of cultures to OPs in situ, the cells were rinsed with saline, removed from the flask by scraping with a rubber policeman, homogenized in a 7 ml dounce homogenizer (Wheaton Glass) for 20 strokes, and assayed for NTE according to the method of Johnson (17). This method is a differential assay requiring the two OPs, paraoxon and mipafox, to define the amount of NTE present. Paraoxon (50uM) is added to all test samples except for a control, to inactivate irrelevant esterase activity. Mipafox (200uM) is then added in addition to paraoxon to a test sample. Inhibition of esterase activity beyond that inhibited by paraoxon alone, yields the basal level of NTE activity. To evaluate other OPs for NTE inhibition, they are added in place of mipafox and after the addition of paraoxon. The inhibition by a test OP beyond that due to the paraoxon is defined as inhibition of NTE.

In 1975, Johnson (18) found phenyl valerate to be a very specific and sensitive substrate for measuring NTE and this substrate has become the substrate of choice. Esterase activity is measured spectrophotometrically at a wavelength of 510nm. Phenol red is formed by the hydrolysis of the substrate; hence, the more activity,

the higher the O.D. 510. Due to the lack of commercial availability of phenyl valerate we sought out a replacement substrate which would be readily available and still serve as a sensitive and specific substrate for NTE. Phenyl 4-chlorobutyrate (Aldrich) was chosen due to its structural similarity to phenyl valerate.

3.3 RESULTS

Using phenyl valerate as substrate and homogenates of non-differentiated neuroblastoma, NTE was found to comprise 25% of the total esterase activity. This figure is 47% of the paraoxon-resistant activity. In an experiment identical to that carried out with phenyl valerate, phenyl 4-chlorobutyrate was used; very similar results were obtained. NTE was found to comprise 20% of the total esterase activity, representing 35% of the paraoxon-resistant esterase activity.

Dose response curves of all OPs tested were identical with both substates (see section of report following this one). Using Homogenates of 74-hour differentiated cultures, NTE was found to comprise 35% of the total esterase activity amounting to 58% of the paraoxon-resistant fraction. This represents a 75% increase of NTE in differentiated cells over non-differentiated cells. While this is significant it is not as dramatic as the 22-fold increase in AChE observed after the same differentiation process (16). The largest NTE fraction was observed using intact cultures and treating them in situ with paraoxon and mipafox (Fig. 1). NTE was found to account for 43% of the total esterase activity, representing 66% of the paraoxon-resistant fraction. The OPs listed inhibited NTE in the following manner (Fig. 2.): leptophos 26%, EPN 14%, OMPA 21% and DFP 77%. A figure of 70% inhibition

or greater is indicative of a compound causing OPIDN (11). A side-by-side dose response study of leptophos and leptophos-oxon was performed with intact cell cultures. At concentrations of 50, 100, and 200 uM leptophos inhibited NTE by 10, 17 and 34%, respectively, while inhibition by leptophos-oxon was 97, 100, and 100%, respectively.

3.4 DISCUSSION

The data obtained to date supports the view that a neuroblastoma cell culture assay for the evaluation of the neurotoxic esterase inhibition (and, by extrapolation, delayed neuropathy) potential or organophosphates could be an effective substitute for the hen brain assay tests performed on treated animals or on brain preparations treated after homogenization. This view is based on the following findings.

1. The NTE levels in the cultures approximated those reported for hen brain and spinal cord. When non-differentiated neuroblastoma cell homogenates were used in conjunction with phenyl valerate, NTE was found to comprise 26% of the total esterase activity and 47% of the paraoxon-resistant activity. When homogenates of 74-hour differentiated neuroblastoma cell cultures were used with phenyl 4-chlorobutyrate as substrate, NTE was found to comprise 43% of the total esterase activity and 66% of the paraoxon-resistant fraction. Johnson (19) reported that NTE comprises 16% of the total esterase activity of hen brain homogenates and 64% of the paraoxon-resistant fraction.

2. The toxicity ranking of the organophosphates, based on the cell culture assays, is similar to that which has been reported for whole animals and for the hen brain. The inhibition of NTE caused by the organophosphates in the cell culture assays were as follows: EPN 14%, OMPA 21%, leptophos 26%, DFP 77%. DFP is

the most potent of the inhibitors which were tested in cell cultures and animals (19).

3. An inhibition value greater than 70% in hen brain assays is predictive of a compound which can cause OPIDN (11). Abou-Donia (20) has reported that EPN and leptophos can cause OPIDN in animals, yet their inhibition in vitro was 14 and 26%, respectively. When leptophos exerts its neurotoxic effects in animals, it is metabolically converted to leptophos-oxon (3). The cell culture system lacks metabolic activation capability (21). However, when the toxicity of leptophos and leptophos-oxon were compared at concentrations of 50, 100, and 200 uM, it was found that leptophos inhibited NTE by 10, 17 and 34%, respectively, while leptophos-oxon inhibited NTE activity by 97, 100, and 100%. Hence, with the oxon high toxicity occurs while the parent compound shows low toxicity. Experiments on other pairs of direct and indirect acting compounds are in progress.

4. Six mg of cellular material was required for the cell culture asay. Johnson (19) reported that 6 mg of hen brain is required for the animal assay for NTE.

In order for the neuroblastoma cell culture assay to be accepted as a valid substitute for the hen brain assay, further research will be needed to optimize the assay and to validate the assay with a large number of organophosphates whose ranking with respect to NTE inhibition in animals is known. Among the optimization studies will be those which relate to state of cell differentiation, culture media formulations, substrate concentrations, and cell handling. A coupled in vitro metabolic activation system also must be developed. In order for the cell culture assays to be

predictive of organophosphorus ester-induced delayed neurotoxicity (OPIDN) it will be necessary to extend the validation studies further and establish the correlations beteen the doses which causes NTE inhibition leading to OPIDN and the per cent inhibition of NTE in hen brain and cell cultures. Only at that time can we be assured that we have a useful in vitro alternative to the hen assay.

An alternative to the hen assay is warranted for several reasons, some of which are overlapping. These include factors relating to reproducibility, ecomomics, and the animal rights movement. Among the factors discussed at the Delayed Neurotoxicity Workshop (22) (personal communication) which related to problems with the hen assay are the following: environmental control (temperature, feed, photoperiod, housing, handling and socialization); disease (viral infections, Marek's tumor, mycoplasma); genetic variation (there are over 500 species of chicken and non-variant, healthy populations cannot be otained routinely); nervous system lesion induction in a commercial setting (attenuated live virus vaccines, chemical toxicants and nutritional deficiencies) (23).

Each of these problems are non-existent or readily circumvented and/or controlled in a well-run tissue culture laboratory. Once the assays have been perfected and a repository of cell stocks established, the time span from the decision to test a chemical in vitro to the conclusion of the test should be about one week, five of the days being required for cell proliferation and differentiation.

3.5 SUMMARY

The neuroblastoma cell cultures were found to have levels of neurotoxic exterase activity comparable to hen spinal cord or

brain. Six mg of cellular material was required to perform NTE assays. Using phenyl valerate as substrate and homogenates of non-differentiated neuroblastoma cells, NTE was found to comprise 26% of the total esterase activity. This figure represents 47% of the paraoxon-resistant activity. Phenyl 4-chlorobutyrate was tested for its efficacy as a sustitute for the substrate phenyl valerate. Dose response curves of all OPs tested were identical with both substrates.

Using homogenates of 74-hour differentiated cultures, NTE was found to comprise 35% of the total esterase activity amounting to 58% of the paraoxon-resistant fraction. This represents a 75% increase of NTE in differentiated cells over that found in non-differentiated cells. The largest NIE fraction was observed when intact cells were used and they were treated in situ with paraoxon and mipafox. The OPs which were studied inhibited NTE to the extent specified: EPN 14%; leptophos 26%; OMPA 21%; DEP 77%. In order to determine whether or not the cell culture system's lack of responsiveness to leptophos could stem from its inability to activate leptophos, a side by side dose response study of leptophos and leptophos-oxon was performed with intact cells. At concentrations of 50, 100, and 200 uM, leptophos-oxon inhibited 97, 100, and 100%.

The data obtained to date strongly suggests that the cell culture assay we have developed mirrors the in vivo hen brain assay system which is used for the study of the effect of OPs on NTE. Thus, further validation experiments appear to be warranted and are being undertaken.

FIG. 5. **Esterase fractions from <u>in situ</u> treatment of nondifferentiated NIE-115 using Phenyl-4-chlorobutyrate substrate**

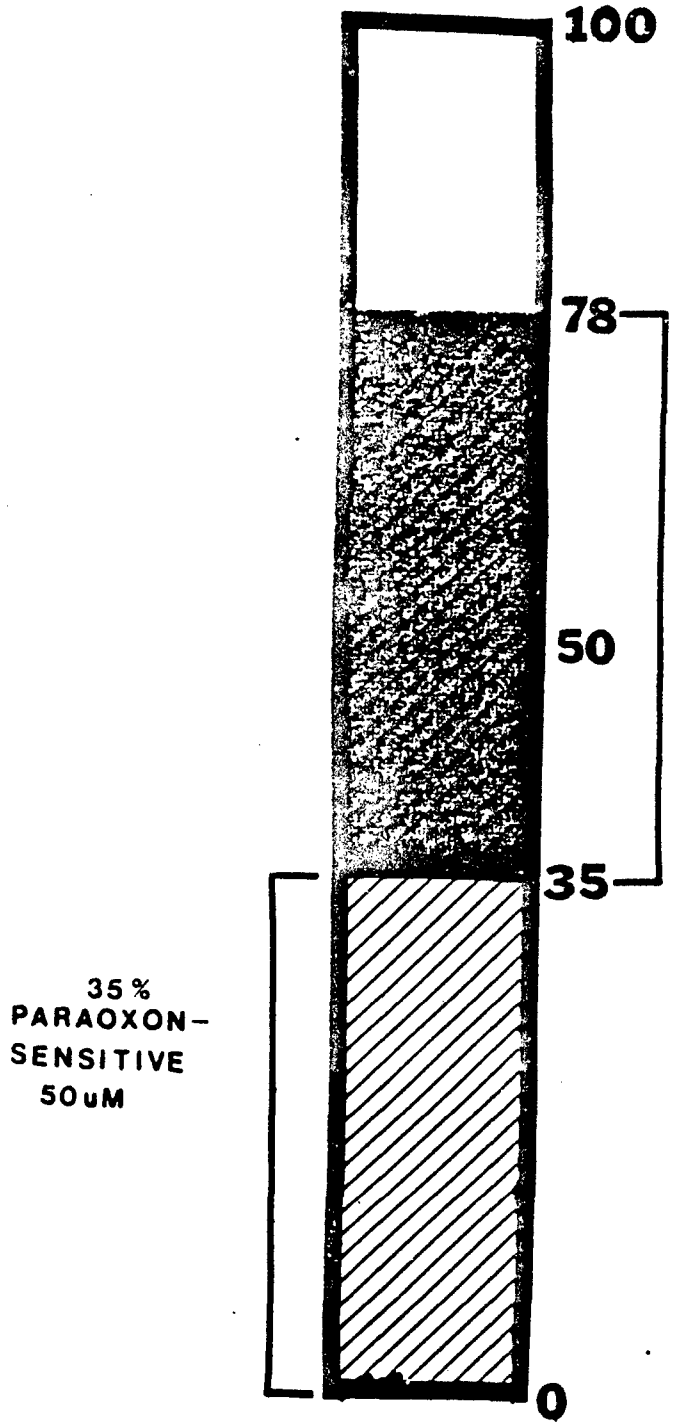
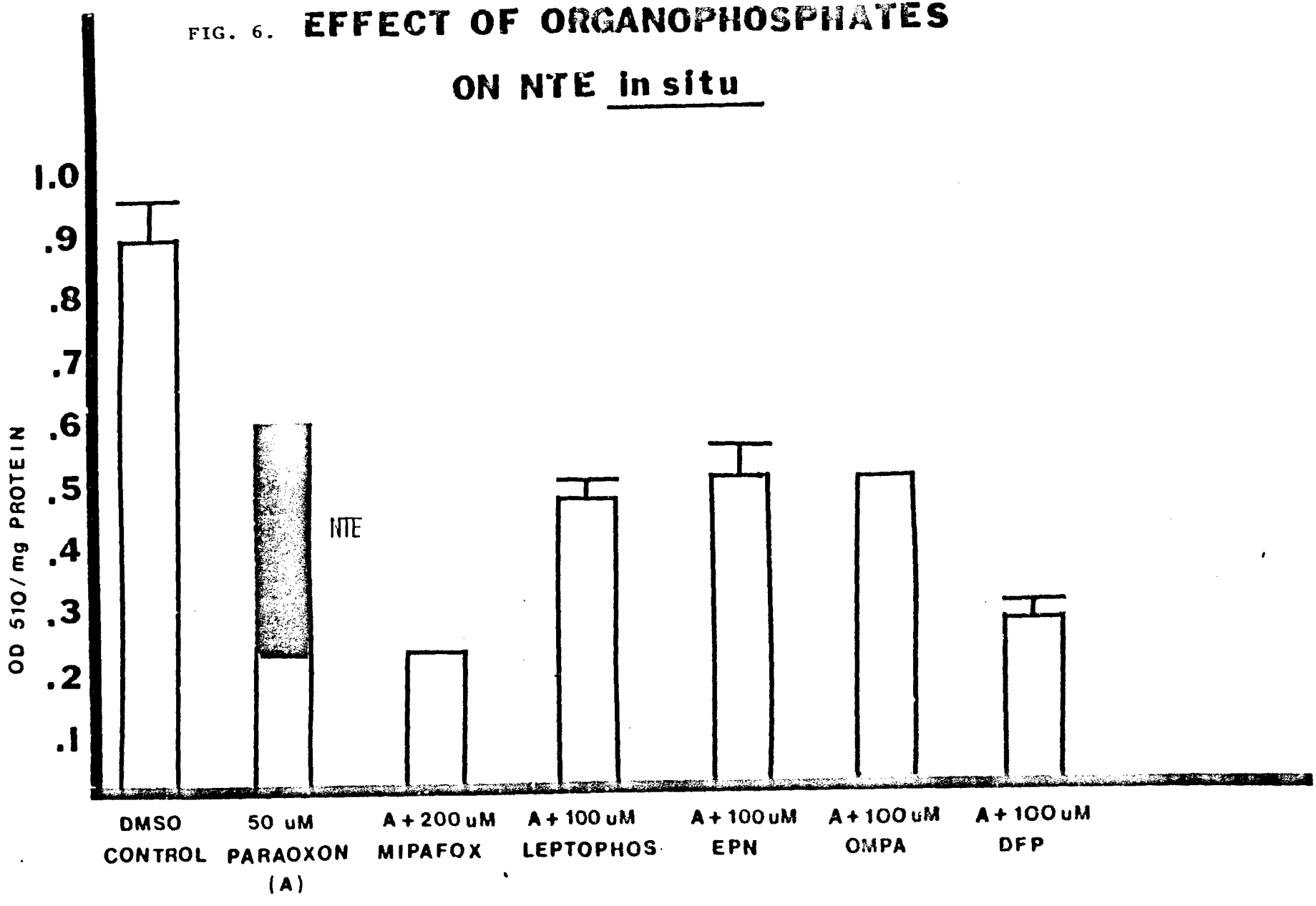


FIG. 6. **EFFECT OF ORGANOPHOSPHATES ON NTE in situ**



4. A Comparative Study of OP-Induced Neurotoxic Esterase Inhibition in Differentiated and Undifferentiated Neuroblastoma Cells with Phenyl Valerate and Phenyl 4-Chlorobutyrate as Substrates

4.1 INTRODUCTION

The purpose of this study was three-fold: 1. to validate in a number of neurotoxic esterase (NTE) inhibition studies the suitability of substituting phenyl 4-chlorobutyrate for phenyl valerate as the substrate in the NTE assay. The former is readily available while the latter, which is the standard substrate, must be synthesized when needed. 2. to determine endogenous levels of NTE and OP sensitivity in differentiated and undifferentiated cultures of NIE-115. Clone N1E-115 can be induced to differentiate morphologically, biochemically, and electrophysiologically by manipulating the culture conditions (Haffke and Seeds, 1975). Thus, it is possible to compare the sensitivity of the same cell in the undifferentiated, proliferative state with the neuron-like differentiated state. 3. to evaluate the use of exposed whole cells rather than cell homogenates in the assay for NTE Inhibition.

4.2 METHODS

This study was an extension of the neurotoxic esterase study reported in the preceding section. Hence, the culture methods and NTE assays were performed as described in that section with the following addition regarding cell differentiation.

The cell cultures were differentiated by withdrawal of serum and addition of 0.5mM dibutyryl adenosine 3^1, 5^1-cyclic monophosphate (cAMP; Sigma) when the cells reached the stationary growth phase. The cells were allowed to differentiate from two to four days prior

to OP exposure.

The OP stocks were prepared at a concentration of 20mM in DMSO and added to whole cultures or homogenates as indicated. The following organophosphate compounds were obtained from the USEPA Analytical Reference Standards Repository, Research Triangle Park, N.C.: O-methyl O-4-bromo-2, 5-dichlorophenyl phenylphosphonothioate (lleptophos), leptophos-oxon, O-ethyl O-4-nitrophenylphenylphosphonothiote (EPN), octamethyl pyrophosphoramide (OMPA), O, O-diethyl O-p-nitrophenyl phosphate (paraoxon). All reference standards were greater than 98% pure. N, N-diisopropylphosphorodiamidic fluoride (mipafox) was kindly provided by M.B. Abou-Donia, Duke University, as was the substrate, phenyl valerate. DFP was obtained from Sigma.

4.3 RESULTS

When EPN, leptophos, mipafox, and paraoxon dose-response studies were undertaken using homogenates of undifferentiated cells and phenyl valerate and phenyl 4-chlorobutyrate as substrates, no substrate-related differences in the overall shapes of the inhibition curves could be discerned (Figs. 7 and 8). The effects of equimolar concentrations (100uM) of the OP on total esterase inhibition are tabulated in Table 9 which shows that the toxicity ranking as well as the overall inhibition of total esterase is not substrate dependent. Furthermore, the same pattern was shown when differentiated cells were used as the source of the homogenate (Fig. 9 and Table 10). The differentiated cells, when compared with undifferentiated cells showed no differences in total esterase activity; however, the NTE fraction increased by 75%, from 20% to 35% (Figs. 10 and 11).

The largest proportion of NTE was seen when intact nondiffer-

entiated neuroblastoma cells were treated with paraoxon and mipafox in situ for 24 hours (Fig. 12). Phenyl 4-chlorobutyrate was used as the substrate. Using this approach, NTE was found to comprise 43% of the total esterase activity. This figure represents 66% of the paraoxon-resistant fraction, which is similar to that reported by Johnson for hen brain. For the purpose of predicting delayed neurotoxic potential of an OP, it is more important to observe the effects of an OP on NTE rather than on total esterase activity. Inhibition values of 70% or greater of NTE by an OP is indicative of a compound which could cause delayed neurotoxicity in vivo (Johnson, 1974). EPN and leptophos at concentrations of 200uM inhibited NTE by 72%. This is a very high dose which would probably cause death by acute cholinergic effects in vivo, thereby preventing observation for OPIDN.

The effect of two doses of EPN and leptophos on NTE activity, using phenyl valerate and phenyl 4-chlorobutyrate are tabulated in Tables 11 and 12, respectively. Again, comparable results were obtained with the two substrates.

There is less inhibition of NTE in differentiated neuroblastoma homogenates by EPN and leptophos at 100 uM than was seen at 50uM using homogenates of nondifferentiated cells (Table 13).

There is a further reduction in NTE inhibition using intact cultures instead of cell homogenates. Again, this may in part be explained by the increased NTE observed (43% versus 35%). In addition, the enzyme may be less available for binding with OPs since it is internalized and the cell membrane presents a barrier to OP penetration. The serum in the medium surrounding the cells also contains protein and esterase activity which may nonspecifically

bind OPs thereby reducing the concentration of free OP available for binding to NTE.

Leptophos and EPN are reported to cause OPIDN in hens (Abou-Donia, 1979). Yet at all but the highest concentration of 200 uM, these compounds inhibited NTE less than 70%, thus indicating a lack of delayed neurotoxic potential. This is not surprising since in order for leptophos and EPN to exert their neurotoxic effects in vivo, they must first be metabolically converted to their oxon forms primarily by the liver enzymes. Thus, while the parent compound leptophos shows low toxicity, the metabolic product leptophos-oxon shows high toxicity (Abou-Donia, 1981). Established cell lines lack metabolic activation capability (Kouri and Schechtman, 1977). In order to determine whether or not the observed results obtained with neuroblastoma may stem from metabolic activation incapability, a side by side dose response study was performed with leptophos and leptophos-oxon using intact 75 cm^2 cultures of nondifferentiated neuroblastoma (Figure 13). In this experiment, the differential toxicity of these compounds was confirmed as was the lack of metabolic activation capability of the cell culture system.

DISCUSSION

The lack of a commercially available source of the substrate phenyl valerate has necessitated that the chemical be synthesized and purified by those who wish to engage in NTE studies. The comparative study with the commercially available substrate phenyl 4-chlorobutyrate, and phenyl valerate indicates that phenyl 4-chlorobutyrate is an appropriate substitute. Thus, those investigators who cannot or choose not to synthesize phenyl valerate, may now enter this important research area free of a dependence on a

specialist in organic synthesis.

SUMMARY

Phenyl 4-chlorbutyrate was tested for its efficacy as a substitute for the substrate phenyl valerate. Dose response curves of all OPs tested were identical with both substrates.

Using homogenates of 74-hour differentiated cultures, NTE was found to comprise 35% of the total esterase activity amounting to 58% of the paraoxon-resistant fraction. This represents a 75% increase of NTE in differentiated cells over that found in non-differentiated cells. The largest NTE fraction was observed when intact cells were used and they were treated in situ to the extent specified: EPN 14%; leptophos 26%; OPMA 21%; DFP 77%. In order to determine whether or not the cell culture system's lack or responsiveness to leptophos could stem from its inability to activate leptophos, a side-by-side dose response study of leptophos and leptophos-oxon was performed with intact cells. At concentrations of 50, 100, and 200 uM, leptophos inhibited 97, 100, and 100%.

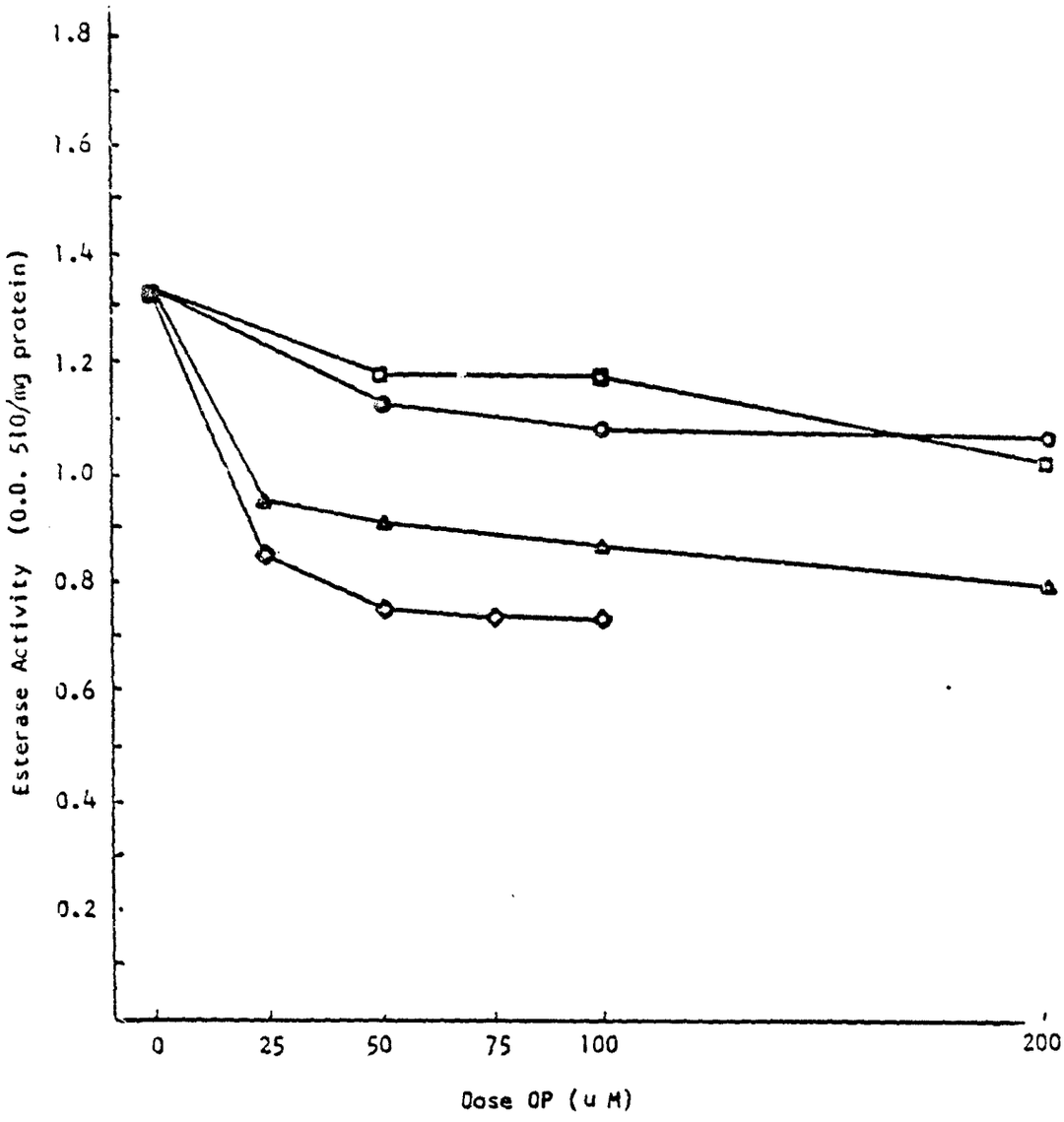


Figure 7 Dose response curves of four OPs on total esterase activity with phenyl valerate substrate using homogenates of non-differentiated neuroblastoma.

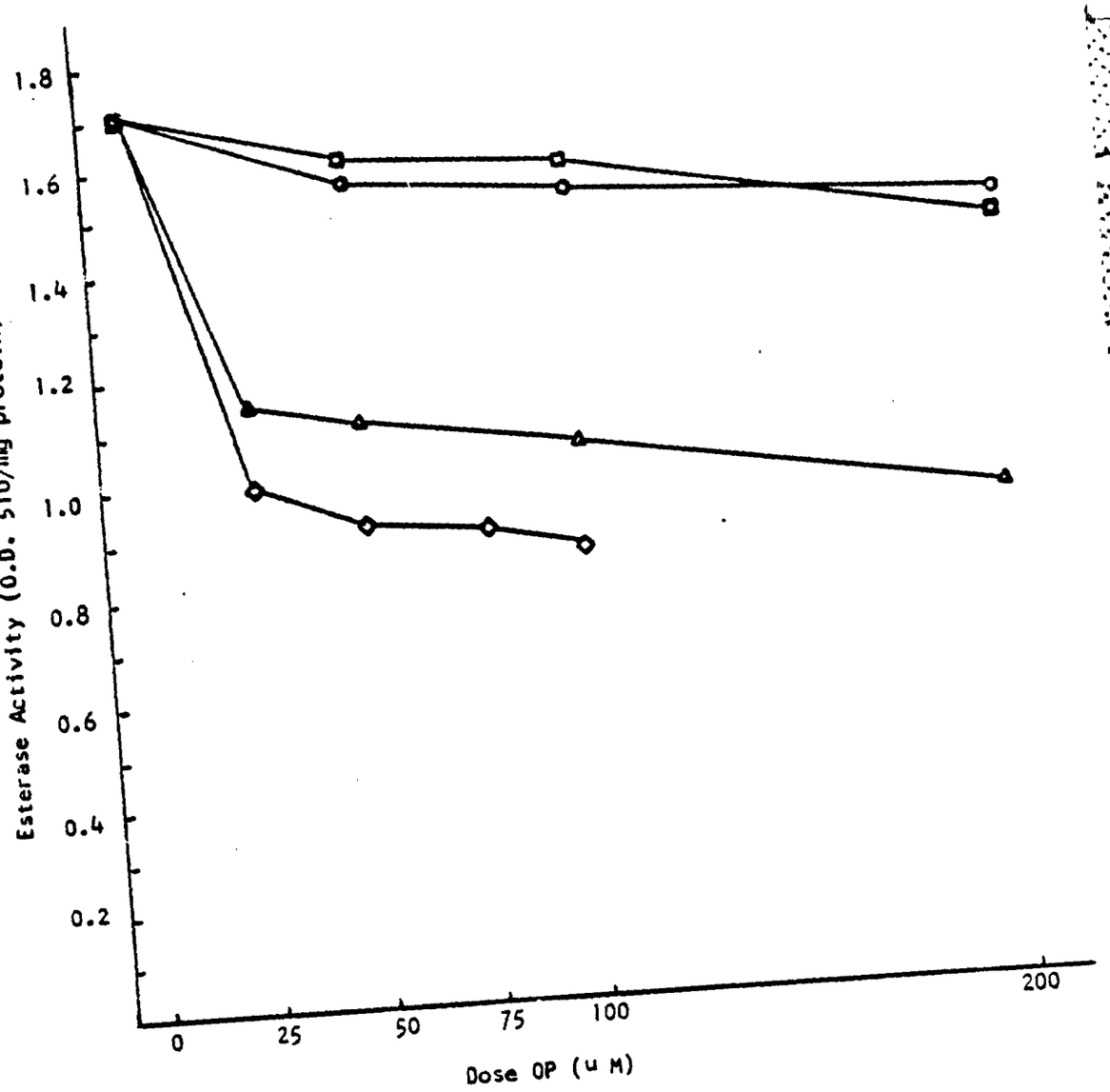


Figure 8 Dose response curves of four OPs on total esterase activity with phenyl 4-chlorobutyrate substrate using homogenates of nondifferentiated neuroblastoma.

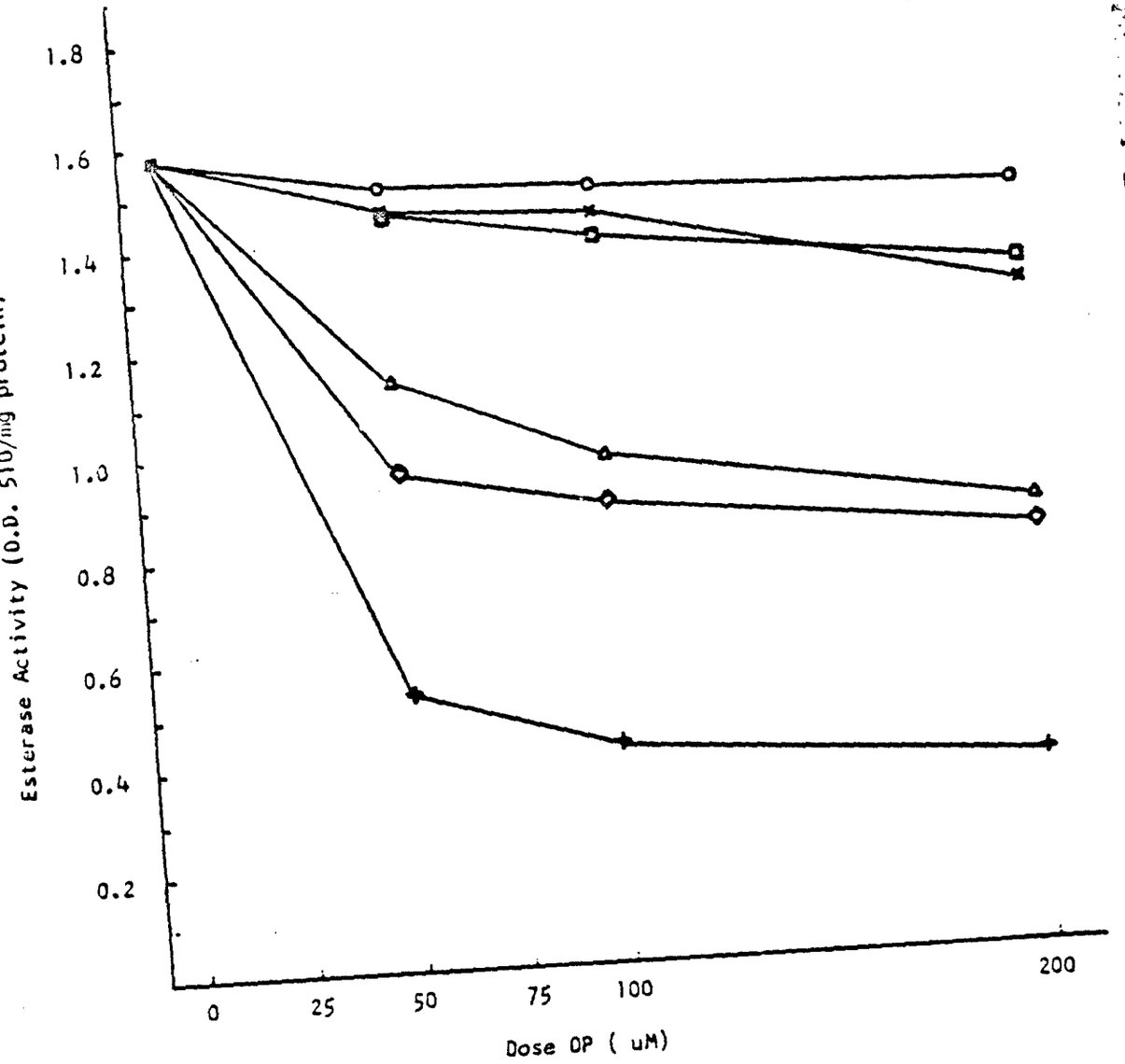


Figure 9 Dose response curves of five OPs on total esterase activity with phenyl 4-chlorobutyrate substrate using homogenates of 74-hour differentiated neuroblastoma.

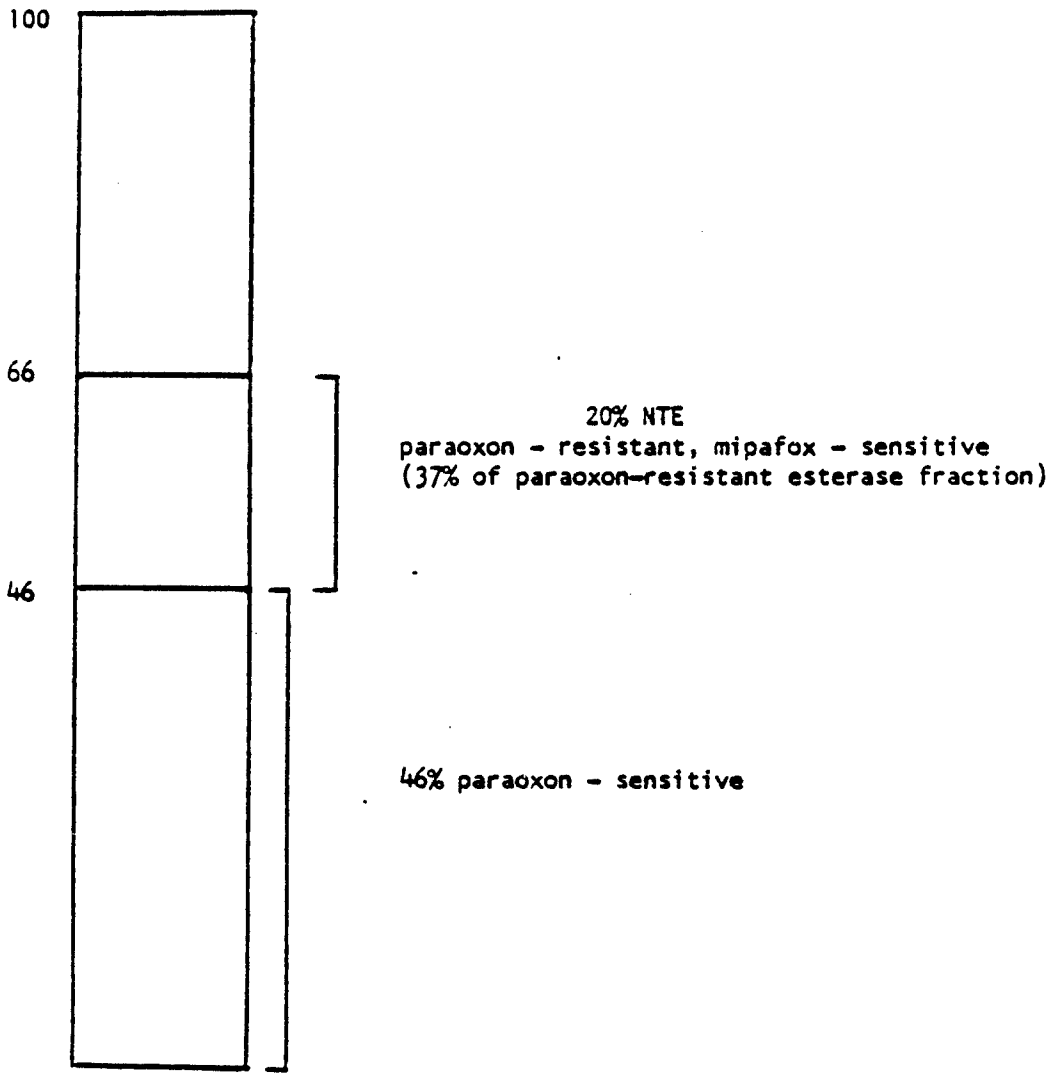


Figure 10 Esterase activity fractions with phenyl 4-chlorobutyrate substrate using homogenates of nondifferentiated neuroblastoma cells.

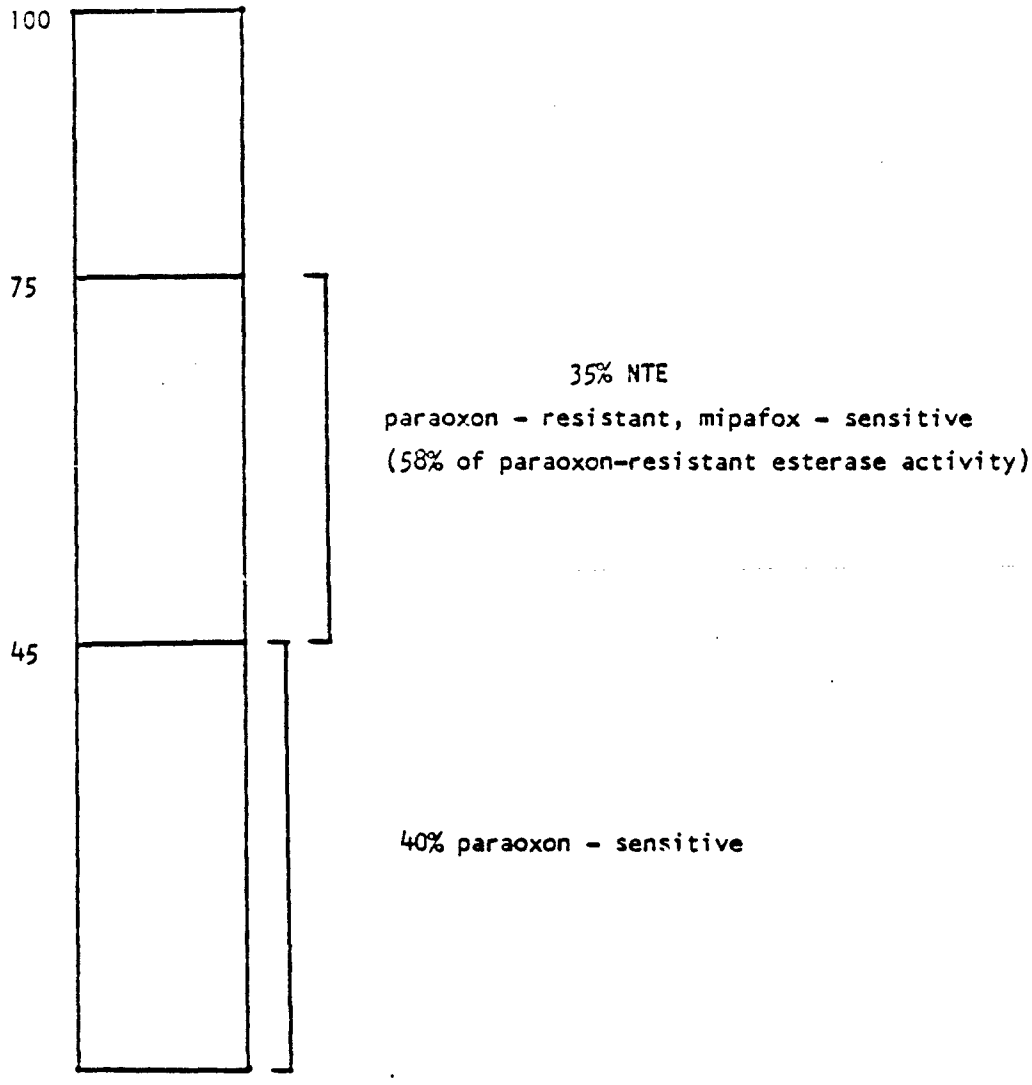


Figure 11 Esterase activity fractions with phenyl 4-chlorobutyrate substrate using homogenates of 74-hour differentiated neuroblastoma.

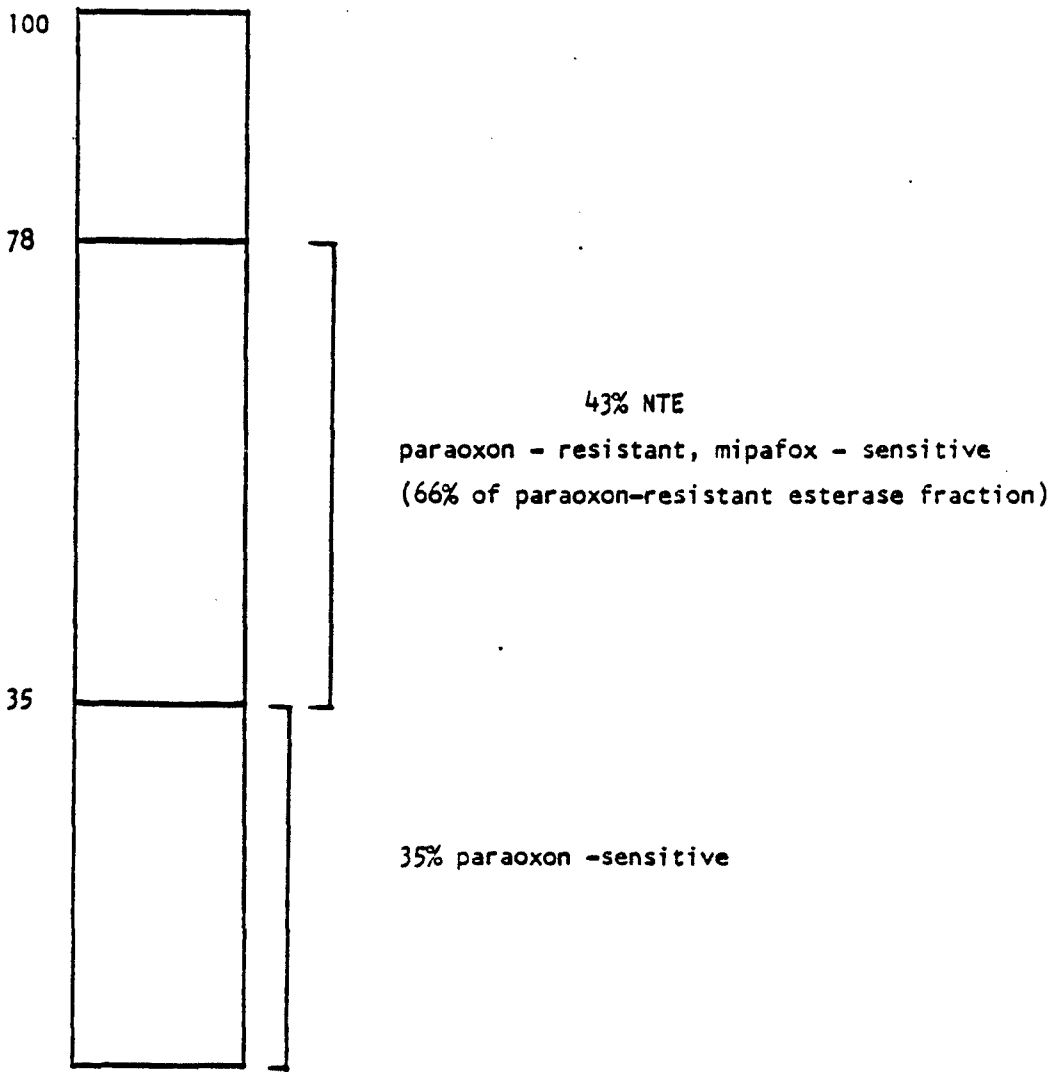


Figure 12 Esterase activity fractions with phenyl 4-chlorobutyrate substrate using intact 75 cm^2 cultures of nondifferentiated neuroblastoma treated in situ.

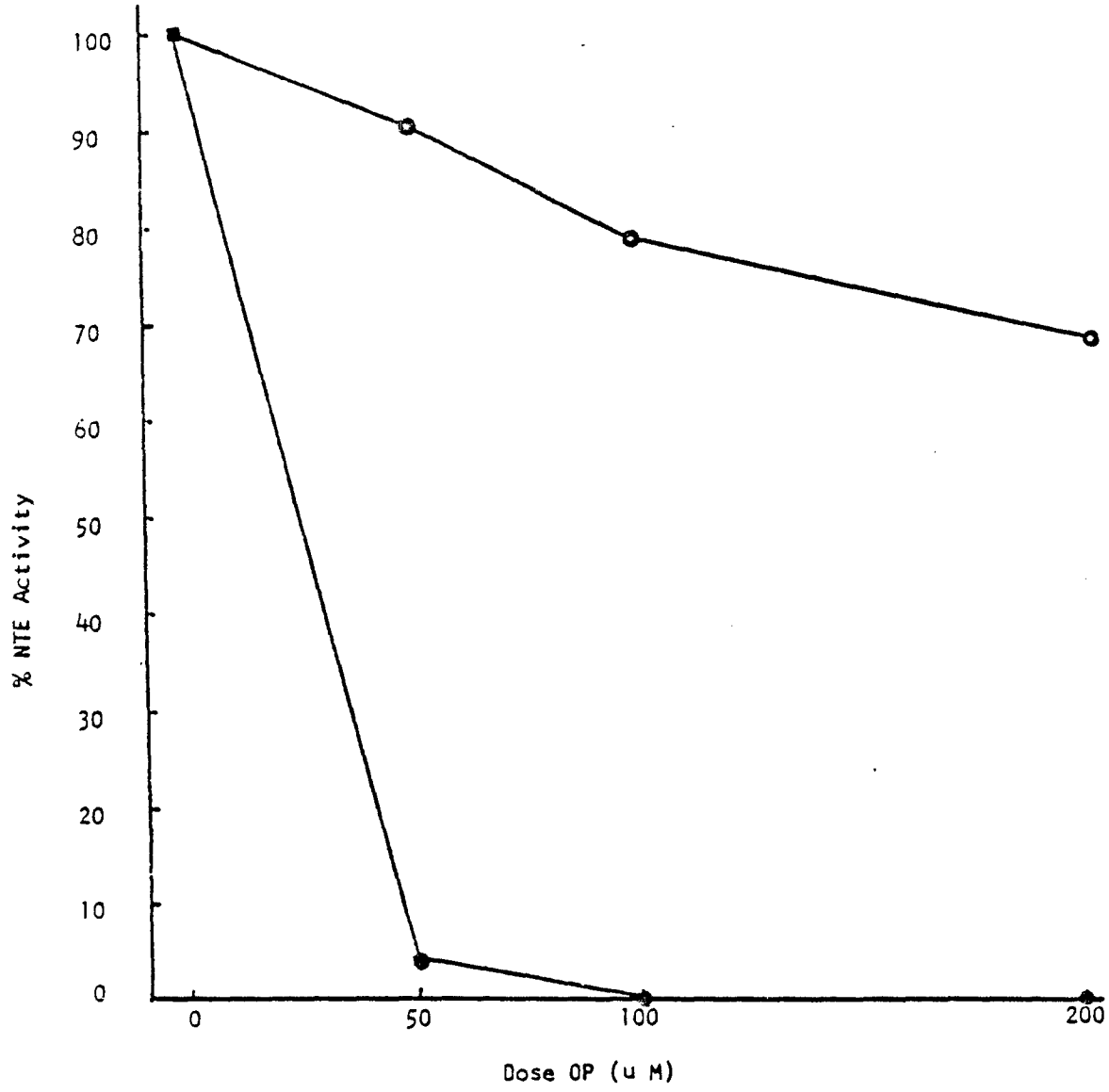


Figure 13 Comparison of the effect of leptophos and leptophos-oxon on NTE activity of 75 cm^2 cultures of nondifferentiated neuroblastoma treated in situ.

Table 9 Comparison of the effect of OPs on total esterase activity using homogenates of nondifferentiated neuroblastoma and the substrates phenyl valerate and phenyl 4-chlorobutyrate.

OP	Concentrations	% inhibition (Total esterase)	
		Phenyl valerate	Phenyl 4-chlorobutyrate
EPN	100 uM	11	11
Leptophos	100 uM	17	13
mipafox	100 uM	36	39
paraoxon	100 uM	45	51

Table 10 Effect of OPs on total esterase activity using homogenates of 74-hour differentiated neuroblastoma and phenyl 4-chlorobutyrate substrate.

OP	Concentration	% inhibition (total esterase activity)
EPN	100 uM	10
leptophos	100 uM	4
mipafox	100 uM	38
paraoxon	100 uM	44
OMPA	100 uM	7
DFP	100 uM	74

Table 11 Effect of OPs on NTE using homogenates of nondifferentiated neuroblastoma and phenyl valerate substrate.

OP	Concentration	% NTE inhibition
EPN	50 uM	52
EPN	200 uM	72
leptophos	50 uM	59
leptophos	200 uM	72

Table 12 Effect of OPs on NTE using homogenates of nondifferentiated neuroblastoma and phenyl 4-chlorobutyrate substrate.

OP	Concentration	% NTE inhibition
EPN	50 uM	60
EPN	200 uM	90
leptophos	50 uM	60
leptophos	200 uM	85

Table 13 Effect of OPs on NTE using homogenates of 74-hour differentiated neuroblastoma with phenyl 4-chlorobutyrate substrate.

OP	Concentration	% NTE inhibition
EPN	100 uM	43
leptophos	100 uM	40
OMPA	100 uM	43
DFP	100 uM	100

Table 14 Effect of OPs on NTE using intact cultures of non-differentiated neuroblastoma treated in situ.

OP	Concentration	%NTE inhibition
EPN	100 uM	14
leptophos	100 uM	26
OMPA	100 uM	21
DFP	100 uM	77

Table 15 Effect of OPs on NTE using intact cultures of nondifferentiated neuroblastoma with phenyl 4-chlorobutyrate substrate.

OP	Concentration	% NTE inhibition
leptophos	50 uM	10
leptophos	100 uM	17
leptophos	200 uM	34
leptophos-oxon	50 uM	97
leptophos-oxon	100 uM	100
leptophos-oxon	200 uM	100

5. Hepatocyte Metabolism of Organophosphates: Effect on Esterase Activity

5.1 INTRODUCTION

The lack of P450 (metabolic activation) activity or its relative paucity in established cell lines limits their usefulness for the evaluation of the toxicological potential of those chemicals which are metabolically activated in the body as a prelude to causing their toxic effects. These same enzymes also cause detoxification of chemicals; hence, many in vitro systems which use established cell lines are not suitable surrogates for in vivo systems which entail detoxification as well as metabolic activation.

This limitation has been reduced or eliminated in bacterial and cell culture assays for induced mutagenesis and carcinogenesis by supplementing the test system with P450-active preparations, including S-9, microsomes, and isolated hepatocytes (Ames, et al 1975; Chu, 1983).

Several organophosphates (OP), such as leptophos and parathion, are relatively non-neurotoxic until they are metabolically converted to their oxon counterparts - paraoxon and leptophos-oxon, respectively. Furthermore, the oxons are capable of being detoxified in the body prior to their excretion.

With this in mind a number of studies were undertaken to establish the basal activation activity and to explore the conditions which might be most suitable for supplementation of a neuroblastoma and chick brain assay in order to have the assay capable of metabolic activation and detoxification. These studies entailed the use of S-9 and rat hepatocyte preparations.

Most studies undertaken in this project entailed comparative

studies with leptophos and leptophos-oxon, with inhibition of total esterase and neurotoxic esterase (NTE) as the end-points. Other OPs also were used in selected experiments.

5.2 METHODS

Total esterase and NTE activity were evaluated using the methods described in the preceding section of this report. The neuroblastoma cell line, N1E-115, and chick brain were prepared as for earlier studies.

S-9 preparations were obtained by centrifugation of rat liver homogenates at 9000 xg. The P450 enzyme activity is microsomal-bound and is in the supernatant fraction. Hepatocytes were isolated from livers of young male rats following perfusion of collagenase through the liver. Such chemical dissociation was followed by mechanical liberation of the hepatocytes (Williams, 1977). The cells so isolated were then washed with PBS and transferred to DMEM or William's medium supplemented with fetal bovine serum.

Any special treatment of the rats or hepatocytes are cited below in conjunction with the results of specific experiments.

5.3 RESULTS

Incubation of neuroblastoma cells or chick brain preparations with S-9 supernatants proved to be toxic to the tester cells. Hence, this approach was abandoned in favor of the use of intact hepatocytes.

The data summarized in Tables 16 to 19 relate to a number of questions which were posed.

1. What effect does preincubation of organophosphates with hepatocytes have on total esterase and NTE activity of neuroblastoma cell homogenates?

About 1.3 x 10^6 hepatocytes were incubated in media prepared without bovine serum albumin and containing the following additives: a) nothing; b) DMSO control; c) 1mM leptophos; d) 1mM leptophos-oxon; e) 1mM EPN; f) 1mM DFP; g) 1mM OMPA; h) 1mM leptophos-oxon but no incubation with hepatocytes. Enzyme activity was assayed at 0, 1 hr., 2 hr., and 3 hr.

The results, summarized in Table 16, show that some detoxification of leptophos-oxon by the neuroblastoma homogenates occurs even in the absence of hepatocytes. As shown on line 3, the percent inhibition became progressively less with time. The rate of detoxification of leptophos-oxon was enhanced by incubation with hepatocytes (line 2). Virtually all inhibition of total esterase and NTE was absent after 1 hour.

The effect of hepatocyte incubation on inhibition by leptophos (line 1) and EPN (line 5) was to enhance inhibition with time. This is interpreted as the consequence of metabolic activation effects exceeding detoxification effects.

No significant changes in DFP and OMPA effects were detected.

2. What effect does inactivation of "oxonase" in the hepatocyte medium have on leptophos and leptophos-oxon inactivation of NTE?

Chick brain homogenates were prepared from 16-17 day old chicks and the effects of leptophos and leptophos-oxon (1mM) were quantified as described earlier with the following exception: 0.2% EDTA was added to the hepatocyte medium to inactivate acetylcholinesterase activity thereby allowing for the accumulation of any metabolically-derived oxons by preventing their breakdown.

The basal level of NTE in the chick brain preparations was 19%. Table 17 shows that during the incubation period, from 45 minutes to 4 hours, the small amount of initial toxicity caused

by leptophos was counteracted. Also, with time, there was a gradual reduction in leptophos-oxon-induced toxicity - from 82% to 68% inhibition of total esterase and from 100% to 89% inhibition of NTE activity.

Table 18 shows the results of a similar experiment except that total esterase and NTE activity determinations were concluded after 20 hours. This preparation showed 100% inhibition of NTE by leptophos-oxon during a three hour exposure period but by 20 hours there was no inhibition of NTE. Total esterase inhibition also declined for the last 17 hours but never reached zero. Leptophos caused no inhibition of total esterase or NTE in this experiment.

3. To what extent are the chick brain and rat brain OP toxicity patterns similar?

Table 19 summarizes the effect of hepatocyte metabolism on total esterase and NTE activity of rat brain homogenates, using 5mM OP concentrations and 0.02% EDTA in the hepatocyte medium. The higher OP concentrations were used because of the high esterase levels in rat brain.

The basal NTE level was 7%. The pattern for leptophos and leptophos-oxon effects indicated detoxification of the OPs throughout the 3 hour exposure period. The results with OMPA are not definitive and may suggest a biphasic activation and detoxification effect. A limited parallel experiment on AChE activity suggested activation of leptophos for enhanced inhibition of AChE (data not shown).

Table 17 shows the effect of leptophos and leptophos-oxon and hepatocyte metabolism on total esterase and NTE of chick brain homogenates.

DISCUSSION

The data strongly suggest that complementation of the cell culture or chick brain preparations with metabolizing hepatocytes could be an effective approach for the in vitro study of the toxicity of those organophosphates which are metabolically activated and/or detoxified. However, the data also points out that the end result which is observed may be the consequence of partially counteracting forces. That is, some potential toxicity may be enhanced by metabolic activation while it may simultaneously be counteracted by detoxification.

Whether or not this is a real problem depends upon the objective of the experiment. The situation described above is similar to the in vivo situation. Nevertheless, with this situation existing, and assuming different and varying rate constants for activation and detoxification, it is imperative that several time points be used for each experiment and the pattern for each OP and end-point be established. This caveat is underscored by our observation that the effect of hepatocyte metabolism on leptophos oxon toxicity of NTE and AChE were not similar nor were the effects on total esterase and NTE parallel to one another at all time intervals.

S-9 supplementation was abandoned in this study because of its toxicity. However, the relative convenience of using S-9 preparations rather than hepatocytes, and the existence of reliable commercial sources of standardized preparations of S-9 suggests that additional studies are warrented in order to try to optimize S-9 use and minimize or compensate for its toxicity. Hepatocyte preparations are quite variable due to the following: there is great variation in P450 induction response within populations of

rats, including litter mates; the isolation of hepatocytes does not give a reproducible product in respect to cell number and viability; P450 activity declines rapidly in vitro. Hence, especially with long-term experiments (3 or more hours), the metabolic activation detoxification potential of the hepatocyte preparations is constantly declining and is doing so at an unpredictable rate.

Table 16

EFFECT OF HEPATOCYTE METABOLISM ON PERCENT INHIBITION OF TOTAL AND NEUROTOXIC ESTERASE ( ) ACTIVITY IN NEUROBLASTOMA CELL HOMOGENATES (OP CONCENTRATION-1mM)

OP	Exposure Time		
	T 0	T 1 hour	T 3 hour
Leptophos	0 (0)	9 (0)	21 (0)
Leptophos-oxon	52 (100)	6 (0)	0 (0)
Leptophos-oxon (no hepatocytes)	60 (100)	50 (75)	28 (0)
DFP	18 (0)	26 (0)	27 (0)
EPN	11 (0)	12 (0)	25 (0)
CMPA	15 (0)	13 (0)	20 (0)

Table 17

EFFECT OF HEPATOCYTE METABOLISM ON PERCENT INHIBITION (BEYOND PARAOXON) OF TOTAL AND NEUROTOXIC ( ) ESTERASE ACTIVITY IN 17-DAY CHICK BRAIN HOMOGENATES. (OP CONCENTRATION-1mM)

Time (min)	Leptophos	Leptophos-oxon
T 0	14 (11)	82 (100)
T 45	0 (0)	82 (100)
T 90	4 (0)	79 (100)
T 120	0 (0)	71 (95)
T 180	0 (0)	71 (95)
T 240	0 (0)	68 (89)

Table 18

EFFECT OF HEPATOCYTE METABOLISM ON PERCENT INHIBITION (BEYOND PARAOXON)OF TOTAL AND NEUROTOXIC ( ) ESTERASE ACTIVITY OF CHICK BRAIN HOMOGENATES. (OP CONCENTRATION-1mM)

Time (min)	Leptophos	Leptophos-oxon
T 0	0 (0)	64 (100)
T 15	0 (0)	64 (100)
T 30	0 (0)	52 (100)
T 45	0 (0)	45 (100)
T 60	0 (0)	42 (100)
T 75	0 (0)	39 (100)
T 90	0 (0)	42 (100)
T 105	0 (0)	42 (100)
T 120	0 (0)	48 (100)
T 150	0 (0)	45 (100)
T 180	0 (0)	45 (100)
T 20 hr.	0 (0)	21 (0)

Table 19

EFFECT OF HEPATOCYTE METABOLISM ON TOTAL AND NEUROTOXIC ( ) ESTERASE ACTIVITY OF RAT BRAIN HOMOGENATE (OP CONCENTRATION-5mM)

Time (hr)	Leptophos	Leptophos-oxon	OMPA
T 0	10 (0)	36 (100)	7 (0)
T 0.5	0 (0)	29 (100)	7 (0)
T 1	0 (0)	19 (57)	39 (100)
T 2	0 (0)	7 (0)	17 (29)
T 3	0 (0)	10 (0)	7 (0)

6. Muscarinic Receptors: Effect of Leptophos, Leptophos-Oxon and Paraoxon

6.1. INTRODUCTION

The in vitro systems used in earlier parts of this project, namely neuroblastoma cells and chick brain, were studied for their suitability for the evaluation of OPs on muscarinic cholinergic receptors. This was deemed feasible in view of the presence of acetylcholine receptors and acetycholine-related enzymes which characterize these preparations (Nelson, 1977).

6.2 MATERIALS AND METHODS

The neuroblastoma cell line, N1E-115, and chick cerebrum and cerebrum organ cultures were used for the receptor binding studies. The method used for determination of the association rate for binding to muscarinic receptors and for the determination of receptor number was based on the approach used by Hedlund and Shepherd (1983). The method entails the use of 3-^{3}(H) Quinuclidinyl benzilate (QNB) as a ligand. Specific binding was athe difference in binding in the absence and presence of 0.01mM atropine.

In order to demonstrate that the doses of OP selected for use were toxic, acetylcholine esterase (AChE) determinations were also made.

The biological material was prepared as described in other sections of this report. Isolated cerebrum was maintained as organ cultures for up to three days.

6.3 RESULTS

The basal levels of specific binding of QNB by neuroblastoma, and 18-day chick cerebrum were 0.0135 pmoles and 0.162-0.713 pmoles, respectively.

The outcome of 12 experiments are summarized in Table . The variables to be noted are as follows: days in culture; with or without OP; specific OP utilized.

In each instance expected levels of AChE were observed. Leptophos-oxon and paraoxon were strongly inhibitory while leptophos caused little or no AChE inhibition.

A cursory review of the last column, which shows specific binding of QNB, suggests that OP-treatment has no consistent effect on acetylcholine receptors in the chick brain. However, when one compares oxon-treated with others and when one looks at changes with time, the following pattern is suggested. There is an increase in receptor quantity with culture time. Furthermore, the quantity of receptors is greatest in those preparations cultured in the presence of the oxons. Such data has been excerpted from Table and rearranged in Table .

6.4 DISCUSSION AND SUMMARY

The low receptor number in N1E-115 cultures makes them less than ideal for studies on receptor effects. NG 108 cell line remains to be investigated in this regard.

The specific binding of QNB by chick cerebrum is similar to that reported by Siman and Klein (1979) for 21 day whole chick brain homogenates (0.140 pmole/mg protein) and that which we obtained for whole mouse brain (1.009 pmole/mg protein). Hence, it appears that the chick cerebrum organ culture could be used as an in vitro system for the evaluation of effects on the ACh receptor.

The increase in receptor number as a function of culture time may reflect late neurological developmental processes. The higher receptor numbers in the OP-treated cultures may reflect a regulatory accomodation stemming from the effect of the OP on acetylcholine

metabolism and/or receptor induction (Siman and Klein, 1979).

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Siman, R.G. and W.L. Klein 1979. Cholinergic activity regulates muscarinic receptors in central nervous system cultures. Proc. Nat. Acad. Sci. 76: 4141-4145.

Table 20. Effect of OPs on acetylcholine receptors in neuroblastoma and chick brain cultures.

Cell/Tissue and Conditions	Units AChE/ mg Protein	Specific Binding p moles ^{3}HQNB Bound
1. Mouse Brain-Fresh	0.61	1.0089
2. NIE Cells-Undifferentiated	0.426	0.0184
3. NIE Cells-72-Hr. Differentiated	1.42	0.0135
4. Chick Cerebrum; Fresh	0.76	0.1616
5. a) Chick Cerebrum; Frozen	0.86	0.1984
b) Chick Cerebrum; 24-Hour Culture, Frozen	1.72	0.3375
c) Chick Cerebrum; 24-Hour Culture; 100 µM Paraoxon; Frozen	0.19	0.4346
d) Chick Cerebrum; 72-Hour Culture	0.57	0.5908
e) Chick Cerebrum; 72-Hour Culture; 100 µM Paraoxon	0	0.7130
6. a) Chick Cerebrum; No Culture	0.480	0.1756
b) Chick Cerebrum; 24-Hour Culture	1.589	0.3016
c) Chick Cerebrum; 24-Hour Culture; 100 µM Leptophos	1.818	0.3937
d) Chick Cerebrum; 24-Hour Culture; 100 µM Leptophos-Oxon	0.109	0.4890
7. a) Chick Cerebrum; 72-Hour Culture	2.780	0.5908
b) Chick Cerebrum; 72-Hour Culture; 100 µM Paraoxon	0	0.7130
c) Chick Cerebrum; 72-Hour Culture; 100 µM Leptophos	0.333	0.3926
d) Chick Cerebrum; 72-Hour Culture; 100 µM Leptophos-Oxon	0.276	0.7129

Table 21. The effect of culture time and OP exposure on ACh receptors in chick brain cultures.

From Experiment 5	%Increase
Increase in specific binding as a function of culture time	
24 hours	41%
72 hours	66%
Increase in specific binding as a function of paraoxon exposure	23%
Increase in specific binding as a function of paraoxon exposure and culture time	73%
From Experiment 6	
Increase in specific binding as a function of culture time (24 hours)	42%
Increase in specific binding as a function of OP exposure:	
to leptophos	23%
to leptophos-oxon	39%
Increase in specific binding as a function of OP exposure and 24 hour culture time	
to leptophos	55%
to leptophos-oxon	64%

7. An In Vitro System for the Evaluation of the Efficacy of Protective Agents: Anti-Paraoxon Monoclonal Antibodies

7.1 INTRODUCTION

Having demonstrated the suitability of the mouse neuroblastoma cell line NIE-115 for the evaluation of acetylcholinesterase (AChE) inhibition by neurotoxic chemicals, it became evident that such a system might be useful for the evaluation of the efficacy of various protective chemicals, including monoclonal antibodies (MAB). The following advantages of such a system were envisaged: 1. ease of handling and quantitation; 2. conservation of materials in limited supply due to cost or availability; 3. reduced use of toxic chemicals; 4. comparative evaluations of different agents or batches under identical experimental conditions; 5. the system would permit the introduction of several variables which could lead to optimization of conditions for protection; 6. the system would allow for studies of physiological/biochemical/cellular environmental studies which bear on the protection process.

The availability of an anti-paraoxon monoclonal antibody (anti-PMAB) afforded us an opportunity to undertake studies on optimization of MAB protection and to evaluate the specificity of the antibody.

7.2 METHODS

A monoclonal antibody was prepared by A.M. Brimfield and K. Hunter (Department of Pediatrics, Uniformed Services University for the Health Sciences, Bethesda, MD), against an analog of paraoxon (phosphoric acid, p-aminophenyl, diethyl triester) conjugated to keyhole limpet hemocyanin (KLH). The paraoxon-KLH

was injected into Balb/C mice and after an immune response was elicited the animals were sacrificed and the spleen cells were fused to P3x63AG8.653 plasmacytoma cells. Hybrid colonies were tested for antibody production using an ELISA assay. Positive colonies were selected for cloning and a clone BD-1 was developed and expanded. kThe monoclonal antibody it produced was designated as anti-PMAB, BD-1.

For the research described herein, ascites fluid containing the monoclonal antibody was processed as follows. The protein was precipitated with 50% saturated ammonium sulfate solution, dialyzed and further purified using a column of BSA-paraoxon bound to a glutaraldehyde silicate matrix (Boehringer-Mannheim Biochemicals, Indianapolis, IN). The purification was carried out according to the method used by Brimfield et al (1985). After purification the antibody was concentrated into a 3-5 ml volume using an Amicon ultrafiltration unit and 43 mm PM10 diaflo ultrafiltration membrane (Amicon Corp. , Danvers, MA). Protein determinations were made on the concentrated antibody using the Bradford method (Bio-Rad, Richmond, CA). The molarity of the concentrated antibody was calculated using 150,000 as the molecular weight for a typical IgG. Verification of the binding ability of the anti-PMAB was established through the use of an enzyme immunoassay (Hunter et al, 1982).

The protective action of the prepared antibody was tested by exposing neuroblastoma cells in culture to paraoxon and anti-PMAB and measuring the activity of the enzyme AChE. The neuroblastoma cells, strain NIE-115, were of mouse origin and were obtained from Marshall Nirenberg (NIH, Bethesda, MD). The cells were grown in Dulbecco's Modification of Eagle's Medium (DMEM) supplemented with

glutamine, bicarbonate, and fetal bovine serum (10%) (Flow Laboratories, McLean, VA). Incubation was at 37^0 C in an atmosphere of 5% CO_2 -95% air. No antibodies were used. A rigorous quality control program involving media and serum evaluation and testing for molds, bacteria, and mycoplasma was used throughout.

As a prelude to an experiment, stock cultures were grown to 75% confluency in $75cm^2$ plastic tissue culture flasks (Corning). The cultures were then subcultured into twelve $25cm^2$ flasks. After 2-4 days an experiment could be undertaken using up to 12 flasks of cells derived from the same subcultured population.

The OP compounds used were paraoxon (O,O-diethyl O-p-nitrophenyl phosphorothioate). These OPs were obtained as standard chemicals from the Analytical Reference Standard Repository (USEPA, Research Triangle Park, NC). The standards were greater than 98% pure and were diluted to 20 mm stock solution and subsequent dilutions were made using serum-free DMEM (SFDMEM) immediately before each experiment. The following safety precautions were taken. When the concentrated OP preparations were handled, gloves and a respirator were used. The waste was collected and allowed to stand for 48 hours in a solution of NaOH at high pH. Dry waste was removed for incineration.

The experiments entailed exposure of the cells to various concentrations of OP alone, antibody preparations alone, and various ratios of different concentrations of OP to antibodies. Appropriate controls (no additions but change to SFDMEM) were used throughout. In preparation for each experiment several preliminary determinations were made. These included a determination of the molar concentration of the lot of antibody being used. In our hands this concentration ranged from 28 to 56 X 10^{-6}M. This antibody concentration then

determined the range of paracxon concentrations to be used. The cells were then exposed to these dilutions and assayed for AChE activity in order to establish what concentration of paraoxon would be grossly inhibitory and yet would permit some AChE activity. Thus, any changes in activity below as well as above the baseline, following antibody addition, could be ascertained. This determination also provided data regarding variation between different lots of paraoxon. Once the anti-PMAB and paraoxon concentrations were determined, all necessary agents were mixed together at the working concentrations in a final volume of 1.5ml. The working concentrations were pre-incubated for 30 minutes at 37^{o}C in microfuge tubes. During the pre-incubation, 25cm^{2} flasks of NIE-115 cells were prepared by rinsing with 10ml of SFDMEM w/20mM Hepes and then by rinsing with 5ml of CMF-PBS (pH 7.4) and aspirating all remaining liquid. The pre-incubated mixtures were then applied to the flasks and exposed to the cells for one hour at 37^{o}C. After exposure to the test mixtures the cells were harvested in 10ml of CMF-PBS and pelleted in 15ml centrifuge tubes at 300xg for 3 minutes. The cell pellets were washed again in 5ml of CMF-PBS and repelleted. The supernatant was decanted and the cells were lysed with 1.5ml of lysis buffer. The lysis buffer was 1x10^{-2} M Tris-HCl, pH 8.8 at 37^{o} C, and 0.1% Nonidet p-40 (Sigma Chemical Co., St Louis, MO). The tubes were vortexed for 15 seconds and placed on ice immediately. The lysates were assayed for protein concentration according to the Bradford Method. The biochemical endpoint of the experiments was the AChE activity in each test sample. The AChE assay was carried out according to the method of Ellman (1981) with the following modifications. The reaction mixture consisted of the following: 2.8ml

of phosphate buffer, pH 8.0; 0.1ml of 2.48×10^{-3}M tetra isopropylpyrophosphoramide (iso_OMPA. Sigma); 0.1ml dithiobisnitrobenzoate (DTNB, Sigma). After the addition of these reagents into a 4.5ml polystyrene cuvette, the cuvette was covered with parafilm and inverted three times. A volume of 0.1ml of cell lysate from one sample was then added to the cuvette, which was again covered and inverted three times. Finally, 0.02ml of the substrate, acetylthiocholine iodide (Calbiochem-Behring Corp., LaJolla, CA), was added to the mixture to begin the reaction, the contents of the cuvette being mixed thoroughly. The cuvette was placed in a spectrophotometer with the wavelength set at 412nm, and readings were taken after 30 seconds and at one minute intervals for the next six minutes. Each sample was assayed in duplicate at 30 ^{o} C. In order to validate the sensitivity of the assay alternate preparations were made for each sample which were identical to test runs except that 0.1ml of phosphate buffer was substituted by 0.1ml of 6.2×10^{-5}M AChE specific inhibitor, 1:5 bis(4 allyldimethyl-ammonium phenyl) pentan -3-one dibromide, BW284C51 (Sigma), (Austin, 1953). The rate calculated for this run represents only background activity (R_B). The most consistent assay results were obtained when all reagents were kept on ice continuously and each sample was mixed immediately prior to being assayed. Calculations were made using the formula of Ellman applied to the parameters of these experimental conditions to obtain a rate of activity expressed as n mole/min/mg protein. The calculated R values were averaged for each sample (Rave) and the background activity (R_B) was subtracted to yield a true rate of AChE activity (RAChE). Resulting AChE values were compared

only within each experimental group of subcultered cells since the level of AChE activity varied for each set.

7.3 RESULTS

Table 23 summarizes the effect of various doses of paraoxon on AChE activity in exposed neuroblastoma cell cultures. The effect was dose-related but non-linear. Exposure to 2.8×10^{-9} M caused about 84% inhibition. This latter concentration was deemed appropriate to use in testing the efficacy of the monoclonal antibody against paraoxon (anti-PMAB). Also shown in Table 22 1 is the protective effect of the anti-PMAB. When the anti_PMAB and the paraoxon were added to the culture simultaneously and at a 100:1 ratio (antibody to OP) about 46% protection was obtained. Preincubation of the antibody and OP together for 30 minutes prior to their addition to the cell cultures, resulted in enhanced but not total protection (74%).

In order to validate the specificity of the observed protection by the anti-PMAB, a non-specific bovine IgG was substituted for the anti-PMAB and used in conjunction with 2.8×10^{-7} M paraoxon (Table 2). The bovine IgG, whether preincubated with the paraoxon or not, provided no protection to the cells. On the other hand, protection was observed again when the anti-PMAB was used, and the degree of protection was enhanced by preincubation (from 53% to 82%). Another specificity experiment, with an IgG of murine origin, was also undertaken and is described later (see Table 5).

The experiments summarized in Table were conducted in the presence of the AChE-specific inhibitor BW284C51, thus allowing for the determination of sample background activity rates (R_B).

These rates varied from 4.5 to 7.0 nmole/min/mg protein in subsequent experiments (Tables 3 to 6). Note that while background activity was always small, its fractional contribution to the total activity increased as the percent inhibition increased.

The experiments summarized in Table 3 were undertaken in order to determine whether or not smaller amounts of the anti-PMAB could be used in the screening studies which were envisaged. Hence, anti-PMAB:OP ratios of 10:1, 25:1, and 50:1 were studied. A 10:1 ratio showed about 15% protection while 25:1 and 50:1 ratios showed 45% and 91% protection, respectively. Hence, it can be concluded that ratios around 25:1 could be used effectively thereby conserving limited supplies of antibody.

Table 24 also shows that bovine IgG used at various ratios affords no protection against paraoxon.

Table 25 shows the protective effect of anti-PMAB when it is used in different ratios with two different concentrations of paraoxon. With a 3.7×10^{-7} M solution of paraoxon virtually no protection was observed, even at a 100:1 ratio afforded 30% protection when the paraoxon concentration was 3.7×10^{-8} M while 250:1 and 500:1 ratios each afforded about 83% protection.

The experiments summarized in Table 25 were developed around the experiments summarized in Tables 23-24. The data shows that a murine IgG, even at a ratio of 250:1, antibody:OP (3.7×10^{-7} M concentration) afforded no protection to the cells. Thus, the notion that the anti-PMAB being used has specificity was reinforced.

In order to evaluate how narrow is the specificity of anti-PMAB, a competition assay involving equimolar amounts paraoxon

and its less toxic analog parathion, was undertaken. Should the parathion compete for the antibody, the degree of protection against paraoxon inhibition should be reduced. The results are summarized in Table 27.

Evidence of cross-reactivity exists. At a anti-PMAB:paraoxon ratio of 250:1, there was about 83% protection of enzyme activity. Addition of parathion, while keeping the paraoxon concentration constant, resulted in a drop to about 54% protection. A similar pattern was revealed when the anti-PMAB:paraoxon ratio was 500:1.

DISCUSSION

Mouse neuroblastoma cell cultures show many neuronal properties including the presence of acetylcholinesterase (Littauer et al, 1978). It has been proposed that a cell line such as NIE-115 could be a useful component of a test battery for the evaluation of the neurotoxic potential of chemicals (Nardone, 1983; Fedali et al, 1982). The experiments reported herein demonstrate the suitability of a neuroblastoma cell line for studies of the effect of organophosphates on acetylcholinesterase activity. The establishment of a concentration-response to paraoxon, in one instance 2.8×10^{-7}M, after a one hour exposure of viable cells substantially reduced the AChE activity to only 16.5% of that of the control cell population. The experiments also support the contention that this in vitro model system for neurotoxicological evaluation also could be used for the evaluation of the efficacy of putative anti-OP protective agents and for optimization of the conditions which afford protection.

It was not suprising to find improved protection with a 30 minute preincubation of anti-PMAB with Paraoxon. The 28%

increase in protection with preincubation in two cases observed, indicated that the antibody and antigen had an affinity for each other which stabilized during the 30 minute preincubation, otherwise pre-incubated samples should not have shown an increase in protection.

Studies on the specificity of the paraoxon for the anti-PMAB over bovine or murine IgG dramatically enforced the idea that protection was dependent upon the monoclonal antibody and not a non-specific phenomenon. The results of the specificity of the anti-PMAB for paraoxon were indicated in the last experiment which used parathion as a competitive antigen seemed to show a degree of affinity of the anti-PMAB for parathion as well as paraoxon. This has been supported by a competitive inhibition enzyme immunoassay, (CIEIA), (Brimfield, 1985), which determined a molar inhibition constant for the binding of the anti-PMAB to paraoxon as well as parathion and several other related OP compounds.

The usefulness of this in vitro model system could be further developed by including an in vitro metabolic activation system such as rat hepatocytes or S-9 microsomal preparations. Other antibodies specific for other OP compounds such as soman and anti-soman MAB (Hunter et al 1982) may be tested under this in vitro system. This system was found to be a useful tool to investigate the mechanism of action of these antibodies in vitro.

The demonstration of in vivo protection against a neurotoxic organophosphate using anti-PMAB (Lenz et al 1984) and the in vivo use of MAB's to protect against the actions of other toxins

such as digitoxin (Haber 1982), accentuates their potential usefulness. The development of an in vitro system to parallel these studies on a cellular level is timely and appropriate, especially when considering the relatively large amount of purified antibody needed for in vivo evaluations and the search for animal alternatives. Also, such an evaluation system may be useful to identify various cellular and extracellular factors which bear on the protective effect of MAB's and their potential uses in therapy.

Table 22. Validation of the Specificity of Paraoxon for anti-PMAB Using a Non-Specific Bovine IgG

Sample	R_{ave}	R_B	R_{AChE}	%Control Activity	%Protection
Control-SFDMEM-HEPES	281.7	7.0	274.7	100%	100%
2.8×10^{-7}M Paraoxon	20.7	4.5	16.7	6.1%	0%
Anti-PMAB + Paraoxon (100:1) Preincubated Separately	158.5	4.7	153.8	56.0%	53.1%
Anti-PMAB + Paraoxon (100:1) Preincubated together	232.0	4.8	227.2	82.8%	81.7%
Bovine IgG + Paraoxon (100:1) Preincubated Separately	17.0	4.5	12.5	4.6%	<0%
Bovine IgG + Paraoxon (100:1) Preincubated Together	20.4	5.3	15.1	5.5%	<0%

Viable neuroblastoma cells were exposed to 2.8×10^{-7}M Paraoxon for one hour in the presence or absence of either anti-PMAB or Bovine IgG. Cells were lysed and assayed for AChE activity. The Antibodies were pre-incubated either separately or along with Paraoxon for 30 min at 37°C. AChE rates were averaged and expressed as R_{ave} in Nm/min/mg protein. In this and subsequent experiments a background rate R_B was subtracted from R_{ave}. The use of AChE specific inhibitor BW284C51 resulted in sample background rates. True AChE rates R_{AChE} were expressed as a % of the control activity. Percent Protection was based on the difference in % Control Activity afforded by the presence of the Anti-PMAB over the control values.

Table 23. Effect of Anti-PMAB-Paraoxon Ratios on Inhibition of AChE Activity

Ratio Antibody/Antigen	Sample	R_{ave}	R_B	R_{AChE}	%Control Protection	
0:0	Control-SFDMEM-HEPES	144.7	7.7	137.7	100%	100%
0:1	2.8×10^{-7}M Paraoxon	24.7	6.8	17.9	13.0%	0%
50:1	Anti-PMAB/Paraoxon	134.8	7.8	127.0	92.2%	91.0%
25:1	Anti-PMAB/Paraoxon	78.6	7.4	71.2	51.7%	44.5%
10:1	Anti-PMAB/Paraoxon	42.6	7.3	35.3	25.6%	14.5%
100:0	Bovine IgG	190.0	9.0	181.0	131.4%	100%
50:1	Bovine IgG/Paraoxon	27.7	8.4	19.3	14.0%	1.1%
10:1	Bovine IgG/Paraoxon	24.0	5.9	18.1	13.1%	0.8%

Viable neuroblastoma cells were exposed to 2.8×10^{-7}M Paraoxon in the presence or absence of different dilutions of anti-PMAB or Bovine IgG. Cells were lysed and assayed for AChE activity. The antibodies except controls were preincubated with paraoxon for 30 min at 37°C. R_{AChE} was calculated for each sample and expressed as percent of untreated control activity. Percent protection was based on the difference in % control activity attributed to the presence of anti-PMAB or test antibody over the control values.

Table 24. Effect of Paraoxon Concentration and Anti-RMAB-Paraoxon Ratios on AChE Activity

Ratio of Antibody to Antigen	Sample	R_{ave}	R_B	R_{AChE}	%Control Activity	%Protection
0:0	Control-SFDMEM-HEPES	278.9	5.4	273.5	100%	100%
0:1	3.7×10^{-7}M Paraoxon$_1$	9.3	4.9	4.4	1.6%	0%
100:1	Anti-PMAB: Paraoxon$_1$	20.8	5.0	15.8	5.78%	4.2%
50:1	Anti-PMAB: Paraoxon$_1$	14.3	4.3	10.0	3.66%	2.1%
25:1	Anti-PMAB: Paraoxon$_1$	16.7	5.0	11.7	4.28%	2.7%
10:1	Anti-PMAB: Paraoxon$_1$	10.9	4.3	6.6	2.41%	0.82%
0:0.1	3.7×10^{-8} Paraoxon$_2$	28.3	4.4	23.9	8.74%	0%
500:1	Anti-PMAB: Paraoxon$_2$	238.2	5.0	233.2	85.3%	83.9%
250:1	Anti-PMAB: Paraoxon$_2$	236.5	5.0	231.5	84.6%	83.1%
100:1	Anti-PMAB: Paraoxon$_2$	103.8	4.8	99.0	36.2%	30.1%

Viable neuroblastoma cells were exposed to one of two concentrations of Paraoxon 3.7×10^{-7}M or 3.7×10^{-8}M, either in the presence or absence of various concentrations of anti-PMAB. Antibodies were pre-incubated along with the paraoxon for 30 min at 37°C. Cells were lysed and assayed for AChE activity. The RAChE was calculated for each sample and expressed as the percent of control activity in the untreated sample. Percent Protection was based on the difference in sample % control activity attributed to the presence of anti-PMAB over the appropriate control activities.

Table 25. Confirmation of the Specificity of Protection by the Anti-PMAB

Ratio of Antibody to Antigen	Sample	R_{ave}	R_B	R_{AChE}	% Control Activity	%Protection
0:0	Control-SFDMEM-HEPES	236.7	6.0	230.7	100%	100%
0:1	3.7×10^{-8}M Paraoxon	18.9	6.0	12.9	5.6%	0%
250:1	Anti-PMAB/Paraoxon	216.6	6.0	210.0	91.0%	90.5%
250:1	Murine IgG/Paraoxon	15.2	6.0	9.2	4.0%	<0%
250:0	Murine IgG	224.2	6.0	218.2	94.6%	94.3%

Viable neuroblastoma cells were exposed to 3.7×10^{-8}M Paraoxon for one hour either in the presence or absence of Anti-PMAB (9.25×10^{-6}M) or Murine IgG (9.25×10^{-6}M). Antibodies were preincubated with paraoxon for 30 min at 37°C. Cells were lysed and assayed for AChE activity. The RAChE was calculated for each sample and expressed as the percent control activity in the untreated sample. Percent Protection was based on the difference in sample % Control Activity attributed to the presence of Anti-PMAB over the activity in control samples.

Table 26. The Effect of Paraoxon on AChE Activity and the Protective Action of an Anti-Paraoxon Monoclonal Antibody

Sample	R(hm/Min/mg Protein	R_{ave}	%Control Activity	%Protection
Control-SFDMEM-HEPES	74.15 74.15	74.15	100%	NA
2.8 x 10^{-9}M Paraoxon	69.53 64.08	66.8	90.1%	NA
2.8 x 10^{-8}M Paraoxon	51.76 52.3	52.05	70.2%	NA
2.8 x 10^{-7}M Paraoxon	12.5 11.95	12.22	16.5%	NA
Control-SFDMEM-HEPES$_1$	74.5 71.4	73.0	100%	100%
2.8 x 10^{-7}M Paraxon$_1$	12.5 12.0	12.3	16.8	0%
Anti-PMAB + 2.8 x 10^{-7}M Paraoxon$_1$ (100: 1) Preincubated Separately	41.1 39.7	40.4	55%	45.9%
Anti-PMAB + 2.8 x 10^{-7}M Paraoxon$_1$ (100:1) Preincubated Together	55.8 58.6	57.2	78.4%	74.0%

Viable neuroblastoma cells were exposed for one hour to Paraoxon in the presence or absence of anti -PMAB. Cells were lysed and assayed for AChE activity. The anti-PMAB was pre-incubated either separately or along with Paraoxon for 30 min at 37°C. AChE rate R was measured and averaged (R_{ave}) and then expressed as percent of control activity. Percent protection was based on the protection afforded by the presence of anti-PMAB and was based on 100 % control activity.

Table 27. Competition of Different Concentrations Anti-PMAB for Two Antigens (Paraoxon and Parathion)

Ratio of Antibody to Antigen	Sample	R_{ave}	R_B	R_{AChE}	%Control Activity	%Protection
0:0	Control-SFDMEM-HEPES	297.8	5.4	292.4	100%	100%
0:1	3.7×10^{-8}M Paraoxon	28.8	4.9	23.9	8.2%	0%
500:0	Anti-PMAB	265.7	5.7	260.0	88.9%	87.9%
500:1	Anti-PMAB/Paraoxon	244.8	5.9	238.9	81.7%	80.1%
500:1	Anti-PMAB/3.7×10^{-8}Parathion	270.6	5.3	265.3	90.7%	89.9%
500:1:1	Anti-PMAB/Paraoxon/Parathion	213.9	5.0	208.9	71.4	68.8%
250:1	Anti-PMAB/Paraoxon	251.7	5.1	246.6	84.3%	82.9%
250:1	Anti-PMAB/Parathion	283.2	4.8	278.4	95.2%	94.8%
250:1:1	AntiPMAB/Paraoxon/Parathion	174.2	6.3	167.8	57.4%	53.6%
100:1	Anti-PMAB/Parathion	51.2	3.9	47.3	16.2%	8.7%
100:1	Anti-PMAB/Paraoxon/Parathion	287.8	5.0	282.8	96.7%	96.4%
100:1:1	Anti-PMAB/Paraoxon/Parathion	45.1	4.7	40.4	13.8%	6.1%

Viable neuroblastoma cells were exposed for one hour to 3.7×10^{-8}M Paraoxon and/cr 3.7×10^{-8}M Parathion in the presence or absence of dilutions of 1.85×10^{-5}M (500:1) anti-PMAB. Antibody was incubated with the Paraoxon and/or Parathion for 30 min at 37°C. Cells were lysed and RAChE was determined then expressed as a %control activity. Percent Protection was based on the protection afforded by the presence of Anti-PMAB from the % Control Activity and is based on 100% control activity. Samples without anti-PMAB are calculated for the sake of comparison.

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8. GENERAL SUMMARY AND CONCLUSIONS

The research reported herein strongly supports the basic premises which prompted this project. Namely, in vitro systems present a good opportunity for the rapid evaluation of the neurotoxic potential of chemicals and such systems, when validated offer the possibility of their adoption for the efficacy of protective agents.

In order to gain maximum benefit from this research effort, further research will be needed to expand the test battery beyond AChE, neurotoxic esterase, total esterase, neuron specific enolase, and total enolase activity. The project should be expanded to include classes of chemicals other than organophosphates and acrylamides and the further development of in vitro methods for the evaluation of the efficacy of prophylactic and therapeutic approaches.